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CYTOLOGICAL ABNORMALITIES PRODUCED BY EXPERIMENTAL TEMPERATURE SHOCK ON TRITURUS TOROSUS EMBRYOS[1]

CATHERINE HENLEY
University of North Carolina

INTRODUCTION

The importance of a complete chromosome complement in normal development is obvious when one considers the evidence that the chromosomes are the carriers of genes governing the characters of the organism. It is of considerable theoretical and practical interest to ascertain the effects of addition or loss of single chromosomes, or of chromosome sets.

Most of the early work in the field of developmental genetics was done with plants, until publication of the reports of Fankhauser (1938 and subsequent papers). These reports indicated the value of a tail-tip method for the cytological study of amphibian larvae. The technique utilized is sufficiently simple that systematic studies can be carried out on a large scale, to determine the incidence of heteroploidy in a natural population of salamander larvae. It was with the view of making such a study on *Triturus torosus* that investigations were begun in 1947 on larvae developing from eggs which had been shipped to Chapel Hill from California, in a mixture of ice and water. These larvae showed a very high incidence of mosaic heteroploidy (43 per cent in a sample of 126 larvae), and of severe mitotic abnormalities of various types. The results of this study will be presented in a separate paper.

The possibility existed that such a frequent occurrence of mosaicism was present in a natural population, as well as in the shipped material. Cytological study of a large group of *T. torosus* larvae collected in nature as young embryos and raised in the laboratory indicated that this was not so (Costello and Henley, 1948, 1949). It was further demonstrated that larvae raised from eggs collected in very cold water had a higher incidence of mitotic abnormality than those collected from warmer water. This finding, coupled with similar work reported by Böök (1943, 1945) and Barber and Callan (1943) implied that low temperature might be at least one of the factors responsible for the effects observed in the shipped embryos. Experiments were therefore planned to test the effects of cold treatment applied at

[1] A dissertation submitted to the Faculty of the University of North Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology.

various stages of development, from a period shortly after insemination through the tail-bud stage, in *T. torosus*.

Some of the data included here were communicated earlier in a preliminary note (Henley, 1948).

The study was carried on under the direction of Dr. D. P. Costello, to whom the author is very grateful for his stimulating interest and his helpful criticisms. The experiments were performed at the School of Biological Sciences of Stanford University during the winter and spring of 1948; it is a pleasure to acknowledge the many courtesies extended during that time by Dr. Douglas M. Whitaker, and Dr. Victor C. Twitty and his co-workers. The accompanying photomicrographs were made by Dr. Costello.

Methods

The embryos used in these experiments were raised in the laboratory at Stanford University from *Triturus torosus* eggs collected at Los Trancos and Ukiah, California, and from eggs laid in the laboratory by female salamanders collected at Ukiah, Portola and Mt. Hamilton. In all cases, the embryos treated in 1948 were raised within a constant temperature range (13 – 15° C.) except during the experimental treatment. Usually, several egg clutches were treated simultaneously, in a large fingerbowl. Shortly before the time of hatching, the egg masses were segregated, so that embryos from a given clutch could be identified. In the 1948 experiments, non-chlorinated Searsville Lake water was used and the larvae were isolated in separate paraffined paper ice-cream cups according to the method described by Costello and Henley (1949). All animals were photographed at the time of the first clipping (Fig. 1). MS-222 (1:2000 in distilled water) was used for anesthetizing the larvae for photography and tail-clipping.

All the 1948 cold treatments reported here involved a temperature shock. Embryos at various stages of development from before cleavage through tail-bud stages were placed in fingerbowls of pre-chilled water maintained at the desired temperature. At the end of the treatment, they were transferred back to the original fingerbowl of water at room temperature and allowed to continue development. The effects of temperatures from 0° (mixture of ice and water) to 8° C. were tested for varying periods of time, as indicated in Table 1. In all cases, the embryos were left within the jelly mass until they hatched normally.

Appropriate controls were kept for the various series; details from the results of a study of these untreated larvae are discussed by Costello and Henley (1949).

When the larvae had hatched and feeding was about to begin, they were photographed and assigned serial numbers. The distal one-third of the tail-tip was clipped, fixed and stained as described by Costello and Henley (1949). After an interval of approximately 12 days, the regenerated tip was likewise amputated and prepared for cytological study. These "second" tips are far superior to "first" tips, since yolk resorption is completed and more mitoses are usually present. All tips were stained with Harris's acid haematoxylin.

Proper feeding is essential if good cytological preparations are to be obtained; an adequate supply of Artemia nauplii was kept in the cups with the larvae to serve as food. These nauplii were cultured in a strong brine solution and washed into fresh lake water before being fed to the salamander larvae.

I am indebted to Dr. Costello for the use of 27 tail-tip preparations of *T. torosus* larvae, made by him in 1942. These tips were derived from embryos which had been cold-treated 5 to 80 minutes after insemination, for periods of 18 to 23 hours. No preparations from newly regenerated tail-tips of this group were prepared and only first clippings are included in the data. The larvae were raised at laboratory room temperature. The cold treatments did not involve temperature shock, the eggs having been chilled gradually and allowed to return slowly to room temperature.

The tail-tip epithelium was studied at a magnification of 210 ×, and any figures of special interest noted on a small "map" of the tip. Using an oil immersion ob-

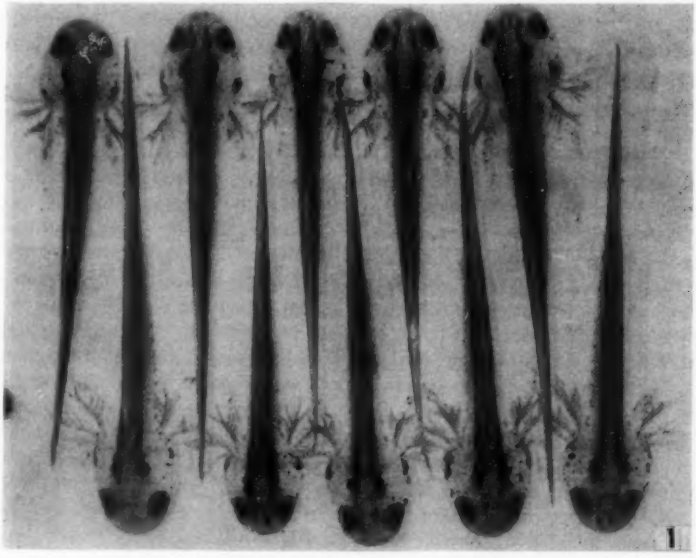

FIGURE 1. A group of *T. torosus* larvae, cold-treated at the gastrula stage for 2 hours at 0° C.; photographed just before their tail-tips were clipped the first time. All ten individuals are normal in general morphology and pigment pattern, but cytologically abnormal to varying degrees. Magnification: 4.5 ×.

jective and 10 × compensating occulars, camera lucida drawings of nuclear size were then made of an area on the dorsal side of each tip, as described by Costello and Henley (1949). Mitotic figures were drawn with the camera lucida at a magnification of 2100 ×, in order to establish chromosome counts.

Three criteria were utilized in classifying the tail-tips: variations in nuclear size or nucleolar number, the presence of abnormal mitotic configurations, and the presence of heteroploid metaphases. The relative value of these criteria has been discussed in the paper of Costello and Henley (1949); they conclude that only decisive chromosome counts of clear mitotic figures are reliable for a definite diagnosis of chromosomal mosaicism, but point out that nucleolar number and nuclear size

TABLE I

The incidence of cytological abnormalities in tail-tip preparations of T. torosus larvae subjected to cold at various stages of development

Stage treated	Treatment	First tips					Second Tips				
		No. tips	Diploid	Variable nuclei	Abnor. mitoses	Mosaics	No. tips	Diploid	Variable nuclei	Abnor. mitoses	Mosaics
10 min. a.i.*	0°–45 mins.	12	4 33%	5 42%	3 25%	0 0	7	0 0	5 71%	2 29%	0 0
Cleavage, blastula	0°–4 hrs.	115	51 44%	49 43%	14 12%	1 0.9%	82	15 18%	28 34%	34 41%	5 7%
Blastula	0°–1 hr.	28	4 14%	11 39%	12 43%	1 4%	20	3 15%	7 35%	6 30%	4 20%
Blastula	8°–3 days; 5°–12 days	10	3 30%	7 70%	0 0	0 0	4	1 25%	0 0	3 75%	0 0
Gastrula	0°–2 hrs.	8	4 50%	2 25%	2 25%	0 0	1	0 0	1 100%	0 0	0 0
Gastrula	0°–2 hrs.	35	10 29%	11 31%	14 40%	0 0	33	5 15%	2 6%	24 73%	2 6%
Neurula	0°–3 hrs.	30	12 40%	13 43%	4 13%	1 3%	3	2 67%	0 0	1 33%	0 0
Neurula	4°–2 hrs.	4	1 25%	3 75%	0 0	0 0	3	0 0	2 67%	1 33%	0 0
Tail-bud	0°–4 hrs.	150	64 43%	55 37%	30 20%	1 0.6%	141	45 32%	62 44%	31 22%	3 2%
Totals		392	153	156	79	4	294	71	107	102	14
Percentages			39%	40%	20%	1%		24%	36%	35%	5%
Controls		582	423	105	53	1	429	284	88	55	2
Percentages			73%	18%	9%	0.17%		66%	20%	13%	0.5%
1942 Material:											
A. 5 min. a.i.*	4°–23 hrs.	5	0	5	0	0					
B. 20 min. a.i.*	0°–1½ hrs.; 4°–19 hrs.	6	1	1	4	0					
C. 30 min. a.i.*	4°–19 hrs.	3	2	1	0	0					
D. 80 min. a.i.*	4°–18 hrs.	13	2	4	2	5					
Totals		27	5	11	6	5					

* "a.i." After insemination.

are often of considerable value as adjuncts to chromosome counts in classifying a given tail-tip. Unfortunately, reliable chromosome counts are often very difficult to make, especially in the experimentally cold-treated material reported here. Early observations on shipped embryos (Costello and Henley, 1947) have indicated that the occurrence of mitoses having a deficiency or a superfluity of single chromosomes may be quite high. Such figures are often crowded, which makes the detection of aneuploid mitoses difficult.

Sometimes tail-tips had more than one type of abnormality; in these cases, classification was based on the most definitely radical of the atypical features. For example, a tip having both marked variation in nuclear size and nucleolar number, and anaphases with lagging elements was classed with the larvae showing abnormalities of mitosis. Similarly, a tip with variable nuclear size, abnormal mitoses and some metaphases having demonstrably more or less than the diploid number of 22 chromosomes (Henley and Costello, 1947) was classified among the mosaic individuals.

Results

The results of a study of 392 "first" tips and 294 regenerated tips are summarized in Table 1, together with the findings obtained from cytological examination of the control tips. No polyploid individuals were observed in either the control or the experimental groups. Among the "first" tips, 153 (39 per cent) were normal diploids, 156 (40 per cent) had nuclei of variable size or abnormalities of nucleolar number, 79 (20 per cent) had abnormal mitoses, and 4 (1 per cent) were chromosomal mosaics. All four of these mosaics appeared to be diploid/triploid individuals, although confirmation by chromosome counts was possible in only one case. In the remaining three, it was obvious that more than 22 chromosomes were present in some of the metaphases. Of the 294 regenerated tips, 71 (24 per cent) were clearly diploid, 107 (36 per cent) had nuclei of variable size or variations in nucleolar number, 102 (35 per cent) had abnormal mitoses, and 14 (5 per cent) were mosaics. Included among the mosaics were four diploid/hyperdiploid individuals, seven diploid/triploids, one diploid/triploid/tetraploid, one possible diploid/tetraploid, and one hypodiploid/diploid/hyperdiploid/triploid. Five of these diagnoses could be confirmed by chromosome counts, and photographic records were made of two more.

Among the "first" tips, the incidence of all types of abnormality was generally lower than among the "second" tips; this was also found to be true of the control animals. The incidence of abnormality in the experimental group was, in almost every case, double or more than double that found among the controls.

The effectiveness of the various treatments in producing mosaicism and other abnormalities is shown in Table 2, where the data are grouped according to the stage treated, without respect to the temperature or duration of treatment. It appears that the early stages of development, through gastrulation, are somewhat more sensitive to the effects of temperature shock than neurulae and tail-buds. This is especially evident in the regenerated "second" tips.

"First" tips from 27 larvae experimentally subjected in 1942 to low temperature were also studied. These had all been cold-treated within 80 minutes after insemination for relatively long periods of time (18–23 hours). Five of the larvae (19

per cent) were normal diploids, 11 (41 per cent) had nuclei of variable size or abnormalities in nucleolar number, 6 (22 per cent) had abnormal mitoses, and 5 (19 per cent) were chromosomal mosaics. Included among these mosaics were three diploid/triploids, one diploid/triploid/tetraploid, and one diploid/triploid/pentaploid. All except a single diploid/triploid were confirmed by direct chromosome counts.

The variation in nuclear size and nucleolar number noted in these experimentally cold-treated larvae was considerably more pronounced, both qualitatively and quantitatively, than in the control tips. It is of interest to note that there was no direct correlation between nuclear size and nucleolar number. Many of the nuclei within the normal range of size had three or even more nucleoli. Conversely, very large nuclei often had only two nucleoli.

In many of the heteroploid and a few of the diploid metaphases the chromosomes were apparently split (Fig. 6). When counts were made of such figures, each pair of chromatids was counted as a unit.

TABLE II

The percentage of cytological abnormalities found in tail-tip preparations of T. torosus larvae subjected to cold at various stages of development

Stage treated	First tips				Second tips			
	2 N	Var. nuc.	Abn. mit.	Mos.	2 N	Var. nuc.	Abn. mit.	Mos.
10 mins. a.i.*	33%	42%	25%	0	0	71%	29%	0
Blastula	38%	44%	17%	1%	18%	33%	40%	8%
Gastrula	33%	30%	37%	0	14%	9%	70%	6%
Neurula	38%	47%	12%	3%	33%	33%	33%	0
Tail-bud	43%	37%	20%	0.06%	32%	44%	22%	2%
1942 Material:								
5 to 80 mins. a.i.*	19%	41%	22%	18%	—	—	—	—

* After insemination.

Cell condensations which appear identical with the lateral line organs were in an atypical position in many of the preparations. Usually, no visible deficiency was present in the normal arrangement, so that the displaced condensations may represent supernumerary lateral line organs.

Blister-like protuberances were occasionally observed on interphase nuclei; in some instances, blebs of chromatin material appeared to have been extruded from nearby nuclei. These blebs retain the organized appearance of normal complete nuclei, and are thus distinct from the nuclear débris described by Costello and Henley (1949). Abnormal nuclei, similar to those observed in control larvae collected from very cold water, were present in some of the experimentally cold-treated tail-tips. Such nuclei are characterized by the presence of a highly basophilic periphery, surrounding a relatively homogeneous central region which stains less intensely.

Among the more striking mitotic abnormalities observed were masses of relatively uncondensed chromatin, sometimes occurring as isolated configurations, and

occasionaly in conjunction with metaphase mitoses. In a few cases, these large masses of basophilic material appeared to be in prophase. An example of this type is shown in Figure 2; here the chromatin mass is connected by a very thin bridge to an adjoining atypical prophase figure, and has many lobes. Three dark bodies (not visible in the photomicrograph) are present in the main mass of chromatin. It is possible that these represent nucleoli, although they do not have the usual appearance of such structures. Normally, nucleoli are not visible in prophase configurations in these tail-tips. In some instances, a chromatin mass in association with a metaphase figure appeared to represent uncondensed chromosomes of the figure, since only a very few normal chromosomes were present.

Metaphase plates occurred in which one or more chromosomes were abnormally oriented on the spindle. Sometimes this abnormality of orientation was so pronounced as to indicate that the chromosome or chromosomes had completely lost spindle connections and were loose in the cytoplasm. Fusion of metaphase chromosomes was apparent in several tail-tips; various steps in the process of fusion were noted, from a stage at which the identity of most of the components was still established to one where only a mass of chromatin was visible. Such chromatin masses are distinguished from the type shown in Figure 2 by the fact that they retain the characteristic ring-shape of a normal metaphase in polar view. Furthermore, the chromatin is much less diffuse than in the unorganized masses of basophilic material. Similar fused configurations were observed in what appear to be telophase (Fig. 9) and anaphase mitoses.

By far the largest number of mitotic abnormalities was observed in anaphases and telophases, confirming an observation made on untreated control material. One of the common types of aberration is shown in Figure 10, where connections persist between several chromosomes of the separating elements. A less radical "sticky" configuration is shown in Figure 11, where only a single connection is present. In the anaphase shown in Figure 12, one of the chromosomes or chromosome fragments appears to be lagging and atypical in its orientation on the spindle. Other examples of this type of abnormality have also been noted. In one case, an apparently normal chromosome is lying at right angles across the spindle; it appears to have lost its connection entirely. Similar examples of "lost" and atypically oriented chromosomes or chromosome fragments are shown in Figures 13, 14, and 18. The fragments may be incorporated into one of the daughter cells as in Figure 13, or lie on the spindle between the two poles as in Figure 14. In the former case, they might contribute to the formation of an aneuploid cell if complete chromosomes are involved. However, in cases where the chromosome material is between the two poles, it seems likely that eventually it would be expelled into the cytoplasm. An abnormal orientation of anaphase poles is shown in Figure 15; one group of daughter chromosomes is lying with the center of orientation almost at right angles to that of the other group. The spindle area of this anaphase shows clearly the characteristic staining properties noted at this stage. Ordinarily, during all stages of mitosis except anaphase and early telophase, only the chromosomes and chromatin material stain blue, leaving the background completely colorless. However, at anaphase, the region between the groups of daughter chromosomes often appears clearly defined as a light blue area. Recognizable spindle fibres have not been definitely identified in the present material using

this staining technique, although they seem to be discernible in some anaphase configurations.

The connections between separating anaphase and telophase groups may persist, so that in some preparations, a high percentage of interphase nuclei with connecting "bridges" is present. Occasionally this connection is thin and attenuated; in other instances it is a rather substantial structure. All gradations between the two extremes were noted, as well as cases where the thin connection appears to have been broken, leaving only a slender projection from each daughter nucleus. Sometimes persistent connections have very sharp bends at the middle, as though some localized pressure were being exerted at that point. In many cases of connected daughter nuclei, the components are asymmetrical, so that one pole is normal in size or larger than normal, while the other is considerably smaller.

In some tail-tips, anaphases or telophases were observed in which nuclear débris, of the type described by Costello and Henley (1949) is present on the spindle or incorporated into one of the daughter nuclei. The characteristic globules of the débris are usually surrounded by a clear halo, which appears well defined against the light blue background of the spindle area.

One remarkably clear tripolar anaphase was found in a tail-tip from a larva cold-treated at the blastula stage (Fig. 16). In this figure, two of the poles appear considerably smaller than the third. The chromosomes in all three groups seem to

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PLATE I

Figures 2–18 are photomicrographs of chromatin configurations in the tail-tip epithelium of *T. torosus* larvae; from whole-mount preparations stained with Harris's acid haematoxylin. Magnification: 1090 ×.

FIGURE 2. A large mass of chromatin which appears to be in prophase. Two lobes are shown connected to the main mass, at the bottom of the photomicrograph. The connection of the lobe at the left is rather thick, while that of the lobe at the right is quiet slender. From a larva treated at the tail-bud stage for 4 hours at 0° C.

FIGURE 3. Normal diploid metaphase in polar view, with 22 chromosomes readily countable. Compare with Figures 4–9.

FIGURE 4. Triploid (or possibly tetraploid) metaphase. The figure is too crowded for an accurate count, but it is obvious that far more than the normal diploid number of chromosomes is present. From a larva treated at the gastrula stage for 2 hours at 0° C.

FIGURE 5. Triploid metaphase. Twenty-eight chromosomes could be counted accurately and more were present. From a larva treated at the blastula stage for 4 hours at 0° C.

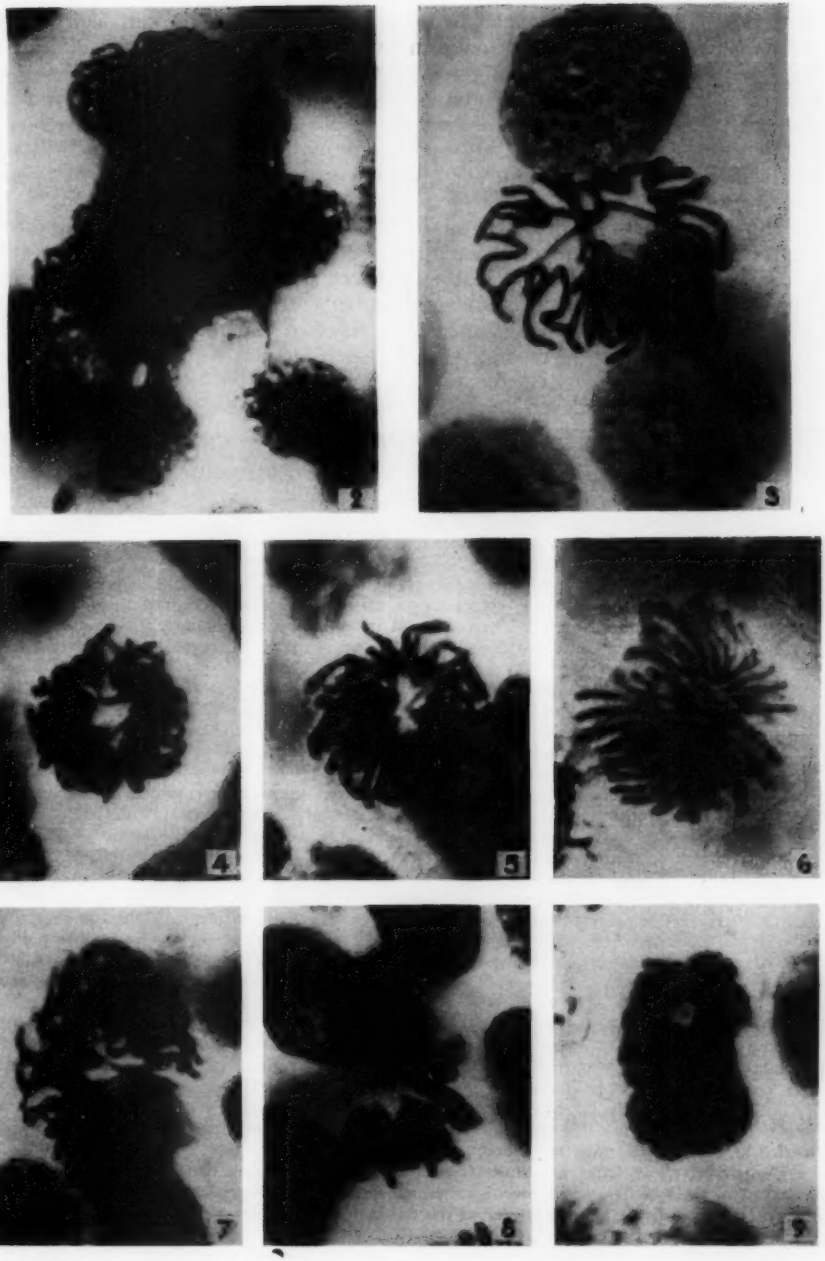
FIGURE 6. Probable tetraploid metaphase. Note the split condition of the chromosomes and the departure from the normal ring configuration of a metaphase in polar view. From a larva treated 80 minutes after insemination at 4° C. for 18 hours (1942 material).

FIGURE 7. Pentaploid metaphase. One sector of the mitosis appears to be on a different level than the remainder, and is therefore out of focus in the photomicrograph. From a larva treated at the blastula stage for 1 hour at 0° C.

FIGURE 8. Hypodiploid metaphase. Thirteen chromosomes were clearly countable, and one or two others could not be traced accurately enough to be included in the count. An adjacent metaphase (not shown in the photomicrograph) is clearly hyperdiploid, with more than 22 chromosomes present. The two figures are apparently in separate cells, although the cell boundaries are not visible. From a larva treated at the tail-bud stage for 4 hours at 0° C.

FIGURE 9. "Arrested" configuration. It is not clear whether this is an atypical telophase or a compound metaphase. Two centers of orientation are present; the chromosomes are beginning to lose their identity and to fuse with one another. From a larva treated at the gastrula stage for 2 hours at 0° C.

PLATE I



be normal. A possible tetrapolar figure is shown in Figure 17, although the abnormal condensation of chromosomes in two of the elements makes it difficult to interpret the relationship of the four poles to one another. The orientation of all four groups toward a common center suggests that this may represent a tetrapolar anaphase. Superficially, the configuration shown in Figure 18 also resembles a tetrapolar mitosis. However, the morphology of the chromosomes in the lower pair of poles indicates that this group of chromosomes is in a different stage of anaphase than the other two.

In general, there seemed to be no correlation between the severity of the cold treatment and the nature of the mitotic abnormalities produced. The greatest number of mosaics (as shown in Table 1) was obtained by treating embryos shortly after insemination, and at the blastula and gastrula stages.

The appearance of the cold-treated larvae is often remarkably normal (Fig. 1), even though the tail-tip preparations from such animals may have quite radical anomalies of mitosis and of chromosome number. This is in accord with unpublished observations made on shipped *T. torosus* and experimentally cold-treated

---

PLATE II

FIGURE 10. "Sticky" anaphase. The groups of daughter chromosomes have begun to separate, but connections persist in at least three regions. From a larva treated at the gastrula stage for 2 hours at 0° C.

FIGURE 11. "Sticky" anaphase. In this configuration only one connection persists between the daughter elements, which have completed or almost completed their movement. The dark granules are embryonic pigment. The outline of the spindle area is faintly discernible in some regions. From the same larva as the metaphase shown is Figure 7, treated at the blastula stage for 1 hour at 0° C.

FIGURE 12. Anaphase with disoriented chromosome or chromosome fragment. The "lost" element appears to be devoid of a spindle attachment; its apparent adherence to the end of one of the daughter chromosomes is unusual in this material. Granules of embryonic pigment are visible in the spindle area, which is stained the light blue characteristic at this stage. From a larva treated at the blastula stage for 4 hours at 0° C.

FIGURE 13. Anaphase or early telophase with chromosome or chromosome fragment "lost" at one pole. The figure is surrounded by a vacuolated area which appears light. Cytokinesis has begun and part of the new cell boundary is visible. From the same larva as Figure 9, treated at the gastrula stage for 2 hours at 0° C.

FIGURE 14. Telophase with two chromosomes or chromosome fragments lagging on the spindle. These appear to be homologous daughter chromosome fragments which have been lost from their respective polar groups. From a larva treated at the blastula stage for 1 hour at 0° C.

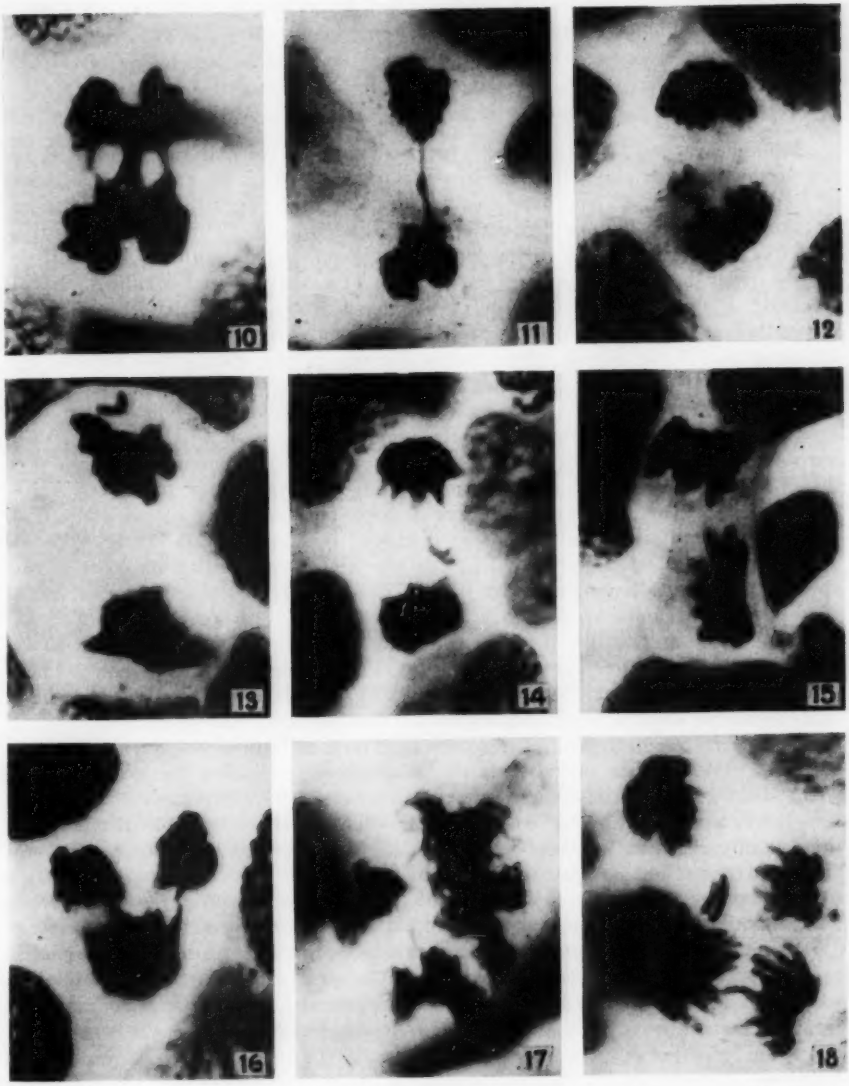
FIGURE 15. Anaphase with bent spindle. The center of orientation of one of the poles is almost at right angles to that of the other. The lightly-stained spindle area characteristic of this stage is unusually clear in this photomicrograph. From a larva treated at the tail-bud stage for 4 hours at 0° C.

FIGURE 16. Tripolar anaphase. This is an exceptionally clear example of a multipolar figure. Two of the poles appear to have fewer chromosomes than the third, but are too crowded to count. The edge of a normal prophase is visible at the right of the photograph. From the same larva as figure 12 treated at the blastula stage for 4 hours at 0° C.

FIGURE 17. Tetrapolar anaphase. The arrangement of the chromosomes in two of the four groups is irregular. All four poles appear to be oriented toward the center. From a larva treated at the blastula stage for 1 hour at 0° C.

FIGURE 18. Two adjacent anaphases with a pair of homologous daughter chromosomes lost from one of the spindles. The chromosome movements have progressed to different degrees in the two figures. From a larva treated at the tail-bud stage for 4 hours at 0° C.

PLATE II



Amblystoma punctatum larvae. In some cases, however, the atypical chromosome condition is reflected in the general morphology. Larvae have also been studied in which all the evidence indicates a normal diploid chromosome condition, even though the animal was abnormal in appearance.

Discussion

A. The absence of polyploidy

Fankhauser and Griffiths (1939) devised an effective and simple method for the production of triploids in *Triturus viridescens*, based on the earlier experiments of Rostand (1934). They found that refrigeration of the eggs shortly after insemination resulted in a high percentage of triploid individuals. This effect was attributed to a suppression of the second maturation spindle which normally gives off the second polar body shortly after fertilization (Fankhauser and Griffiths, 1939). Recently, Fankhauser and Godwin (1948) have demonstrated that the most important effect of heat treatment in producing triploid *Triturus viridescens* eggs is a submergence of the mitotic figure below the egg cortex, giving two haploid egg pronuclei. Fankhauser and Watson (1942) and Briggs (1947) also have found that heat treatment shortly after insemination is effective in producing triploids. It appears that temperature shock is the important factor in all these cases, since abrupt removal of the eggs to and from the experimental temperature bath produces a considerably higher incidence of triploidy than less drastic transfers.

Possible explanations for the absence of polyploidy in a "natural population" of untreated *T. torosus* larvae have been discussed by Costello and Henley (1949). It is suggested that balanced lethal factors are often present in the diploids of this species. Triploids, having an extra dose of one or more of the lethal genes, might die early in development, due to a lethal imbalance. Presumably, a similar explanation could account for the absence of any cases of triploidy among the larvae cold-treated shortly after insemination by the Fankhauser-Griffiths method. There is, of course, no direct evidence for such a theory. Although exact records of mortality were not kept, it is perhaps significant that many of the embryos developing from *T. torosus* eggs treated by this technique died early in development. Indeed, a considerable number of experiments on single egg clutches subjected to cold shock shortly after insemination are not included in this report because the embryos died before a cytological study could be made. The possibility remains that a larger number of cases than that reported here (26) might reveal instances of triploidy after appropriate temperature shock treatment.

Fankhauser (1945) has pointed out that eggs from some *Triturus viridescens* and axolotl females are refractory to cold treatment, and produce predominantly diploid embryos. A similar difference in resistance to temperature shock may exist between species of Triturus. In any event, it is quite surprising that in a total of more than 2200 *T. torosus* larvae (normal, shipped and cold-treated) examined by Dr. Costello and the author, no completely polyploid individuals have yet been found.

B. Mosaicism

As by-products of parthenogenesis, cold, colchicine and heat experiments, and as the direct result of temperature shock later in development than that designed to

produce triploidy, the production of complex mosaics has been reported in the literature over a period of years. These mosaics often possess radical mitotic abnormalities as an accompanying feature.

Parmenter (1933, 1940) found haploid/diploid, diploid/triploid and diploid/triploid/tetraploid individuals in parthenogenetic *Rana fusca* and *R. pipiens* larvae.

In a group of 100 refrigerated *Triturus viridescens* eggs, Griffiths (1941) found one haploid/triploid and five haploid/diploid larvae. Costello (1942) reported two haploid/diploid mosaics in a total of 100 larvae raised from cold-treated *Triturus similans* (granulosus) eggs. Barber and Callan (1943) studied tail-tips of cold- and colchicine-treated *Triton vulgaris* larvae, and described tetraploid mitoses resulting from these treatments. Böök (1943) chilled *Triton taeniatus* eggs later than half an hour after fertilization but before the first cleavage. The duration of treatment is not clearly specified, but appears to have been for periods of 6 to 15 hours. He reports a high mortality in the experimental eggs, only six of sixty-two cases surviving long enough for cytological studies to be made. All six survivors were complex mosaics of various types. In another series of experiments Böök (1945) treated *Triton taeniatus* and *Triton cristatus* eggs 4 to 15 hours at 2° C., more than thirty minutes after insemination. Thirteen embryos of the sixty-four treated survived to the neurula stage, at which time they were examined cytologically. Six of these thirteen cases were complex mosaics. A great variation in nuclear size and a number of mitotic abnormalities were also noted.

Briggs (1947) applied the heat-treatment technique of Fankhauser and Watson (1942) to anuran material. He treated 347 *Rana pipiens* eggs at various intervals after insemination, at temperatures of 35° and 37.5° C. for 4 to 7.5 minutes. Briggs found 32 "mixed" abnormal neurulae (9.2 per cent), which appear to have been haploid/diploid or haploid/triploid mosaics.

Fischberg (1947) subjected eggs of *Triton alpestris* to cold shock within 30 minutes after insemination. Among 553 larvae studied cytologically by the tail-tip method, he observed 27 haploid/diploid, 20 haploid/triploid, 5 diploid/triploid, and 1 diploid/triploid/tetraploid mosaic individuals. Eight larvae were non-mosaic aneuploids, and the remainder were euploid (haploid, diploid, triploid and tetraploid).

In general, the results of a study of the cold-treated *Triturus torosus* embryos are quite comparable to those discussed above. The incidence of chromosomal mosaicism was significantly higher than in the control larvae, although it was not as high as that (43 per cent for a sample of 126 tips) reported for *T. torosus* embryos subjected to shipment (Costello and Henley, 1947, 1948). This is understandable in view of the fact that embryos shipped for a considerable distance in ice and water are subjected to low temperatures for several days. Most of the experimental treatments reported here were not so prolonged. A few tests were made for comparable periods, but the embryos died before they could be tail-clipped. The "D" group of larvae cold-treated in 1942 shows a remarkably high frequency of mosaicism (5 larvae of 13 treated for 18 hours, beginning 80 minutes after insemination). This is a considerably longer period of cold-treatment than the majority of the experiments reported here. The high degree of effectiveness of the cold shock in producing mosaicism in this group of

eggs may be due to the fact that the treatment was operative during an especially susceptible physiological state of the zygote.

The mosaics found in the study of cold-treated embryos were much more radical than those occurring in the "natural population" studied as a control series (Costello and Henley, 1949). This is in contrast to the qualitative similarity of mitotic abnormalities in the control and experimental tips. Two of the three mosaic tail-tips observed among the controls were apparently diploid/hyperdiploid, and the third was diploid/tetraploid. One of the diploid/hyperdiploid tips was from an animal collected and raised under constant temperature conditions. The remaining two were from larvae which had been collected in very cold water. None of these three cases was as markedly atypical as the majority of instances of mosaicism among the experimental larvae. It is probable that none had been exposed to as pronounced temperature shock as the experimentally cold-treated animals. This is interpreted to indicate that there may be a direct relationship between the severity and duration of the cold shock and the degree of cytological abnormality resulting in a given individual. However, Böök (1943) expresses the opinion that no such relationship holds in Triton.

The frequency of mosaicism (especially of aneuploid constituents) may be considerably higher than the data indicate since, as was noted above, many of the metaphases are too crowded for accurate chromosome counts. Evidence for this possibility is afforded by the high incidence of variations in nuclear size and nucleolar number. Böök (1945) also reports a great variation in nuclear size in cold-treated Triton larvae. Since both of these criteria are known to be indices of heteroploidy, it seems quite likely that some of the variations found represent interphase nuclei with varying chromosome numbers.

C. Mitotic abnormalities

There have been a number of descriptions of abnormalities of spindle formation in amphibian nuclei, following irradiation, cold and colchicine treatments. An early report was that of Alberti and Politzer (1924a, 1924b) who observed the effects of x-rays on embryos of *Salamandra maculosa* eight days after insemination. The corneal epithelium of these larvae was studied at varying intervals following irradiation, and a variety of anomalies of mitosis, similar to those reported in the present study, was described. Böök (1943) treated neurulae at 2° C. for 8 hours, and examined the embryos cytologically five hours after the end of the treatment. He observed a tripolar anaphase and other abnormalities of spindle formation. Barber and Callan (1943) described a number of types of mitotic abnormality observed in tail-tips from cold- and colchicine-treated *Triton taeniatus* larvae. Many of these abnormalities were very similar to the ones described in the present study (e.g., "sticking" anaphases).

In the cold-treated *T. torosus* material, the frequency of various types of mitotic abnormality is higher in the experimental tips than in the control. Furthermore, it is appreciably higher in the regenerated experimental tips than in the "first" tips. There is a possibility that cold-treatment has delayed effects on mitosis which are expressed only after the stimulus of clipping has occurred. If this is so, such delayed effects must be operative for a relatively short time, since the incidence of mitotic abnormality in third clippings is apparently not as high as in the second

clippings. This has been found to be the case in tail-tips from shipped embryos, although no data are available for experimentally cold-treated individuals. Briggs (1947) has pointed out that in heat treated *Rana pipiens* larvae, mitotic abnormalities do not show up until the blastula and gastrula stages. A comparable retarded expression of effects might be involved in these cold-treated larvae. There is also the possibility that the higher incidence in "second" tips may be explained by the fact that such preparations usually have more mitotic figures than either "first" or "third" tips, and are generally more favorable for cytological study.

The frequent occurrence of mitotic abnormalities is of considerable interest when compared with that found in tail-tips from a "natural population," although it is lower than the incidence in tail-tips from shipped larvae (unpublished data). In general, the difference is quantitative rather than qualitative, since the types of abnormality observed are comparable to those found in both normal and shipped larvae. The main exception to this statement is found in the occurrence of multipolar spindles in the experimentally cold-treated and in the shipped larvae. No such configurations were observed in control tips; they may be the result of quite radical and prolonged temperature shock, as well as of other factors.

D. The relationship between morphological and cytological characteristics

The normal appearance of many of the cold-treated larvae affords additional evidence for the idea expressed by Costello and Henley (1949) that the general morphology and pigment pattern of a given larva are not necessarily valid criteria of its chromosome condition. Presumably, there is a fairly close correlation between the size of the pigment cells and the number of chromosome sets present in euploid individuals, but this assumption cannot be taken to apply too rigidly. Thus, Fankhauser (1945) points out that such a criterion is not always reliable in the case of axolotl larvae, where the size of the pigment cells in triploids often is not clearly distinguishable from that in diploids. In view of these findings, morphological characteristics must be used with caution as a criterion of heteroploidy. The fact that *T. torosus* larvae abnormal in general appearance may be cytologically normal confirms this belief.

E. General effects of low temperature

It is interesting to speculate on the possible reasons for the special susceptibility of anaphases and telophases in *T. torosus* to the effects of cold. Costello and Henley (1949) have suggested that the lagging and lost chromosomes and chromosome fragments noted may be due to the fact that those chromosomes lacking kinetochores would be left behind in the migration of the daughter elements to opposite poles. Since no such migration has yet occurred at the metaphase, the loss of kinetochores would pass unnoticed unless the chromosomes failed to become located on the equatorial plate. It is also possible that there is a decrease in the adhesive qualities of the chromosomes or of the spindle fibres during anaphase and telophase. This would result in the higher incidence of lagging or lost elements reported at these stages.

Böök (1943, 1945) attributes the effects of cold to a partial or complete paralysis of the spindle apparatus. This might be especially conspicuous at anaphase and telophase, interfering with the normal movements of the chromosomes. He inter-

prets his observed cases of atypical metaphase configurations as being due to the loss of ability of the centromeres to orient themselves properly. Such an explanation of the facts seems rather vague, since it does not account for the actual cause or manner of loss of the capacity of the centromeres for orientation.

It is difficult to ascribe the cytological abnormalities obtained after cold treatment to any specific effect of the low temperature. Böök (1945) interprets the origin of mosaics as a result of the omission of nuclear and cell division after cold treatment, with the retention of the division rhythm of centrosomes and chromosomes. Fischberg (1947) suggests three possible modes of origin of the aneuploid constituents of mosaics: (1) loss of single chromosomes at anaphase; (2) failure of chromosome division; (3) disorderly division of chromosomes in multipolar figures. From the evidence obtained in the present study of cold-treated *T. torosus* larvae, it seems possible that any or all of these factors may be operative in the production of the observed abnormalities. Since the loss of single chromosomes at anaphase appears to occur quite frequently, it may be that this is an especially important factor, as pointed out by Costello and Henley (1949). "Physiological polyspermy" normally occurs in the fertilization of salamander eggs; thus, there is also the possibility that low temperatures affect the supernumerary sperm nuclei so that they contribute to the formation of mosaic areas instead of degenerating as is usually the case.

In general, the evidence from this study indicates that low temperatures may result in the production of chromosomal mosaics and abnormalities of mitosis, similar to those observed in tail-tips from the larvae shipped in ice and water. The relative roles of anoxia, agitation and other factors in the shipped material, however, remain obscure.

Summary

1. A study of the incidence of chromosomal mosaicism and abnormalities of mitosis has been made, utilizing tail-tip preparations made from larvae of *Triturus torosus* subjected early in development to low temperature.

2. Three hundred-ninety two "first" tail-tips and 294 regenerated "second" tail-tips have been studied cytologically from preparations made in 1948. An additional 27 preparations made in 1942 have also been studied. Similar observations were made in 1948 on tail-tips from 582 control *T. torosus* larvae.

3. The experimental larvae were exposed to low temperatures (0 to 8° C.) for varying periods of time (45 minutes to 23 hours) at stages of development from 5 minutes after insemination, through blastula, gastrula, neurula and tail-bud stages.

4. No haploid or polyploid individuals were observed, in either the control or experimental groups.

5. Of the 392 "first" tips, 153 (39 per cent) were normal diploids; 156 (40 per cent) had nuclei of variable size or variations in nucleolar number; 79 (20 per cent) had abnormal mitoses; and 4 (1 per cent) were chromosomal mosaics. Of the 294 "second" tips, 71 (24 per cent) were normal diploids; 107 (36 per cent) had nuclei of variable size or variations in nucleolar number; 102 (35 per cent) had abnormal mitoses; and 14 (5 per cent) were chromosomal mosaics. Of the 27 tips prepared in 1942 from cold-treated animals, 5 were clearly diploid, 11 had nu-

clei of variable size or variations in nucleolar number, 6 had abnormal mitoses, and 5 were chromosomal mosaics. In general, the incidence of all types of cytological abnormality was higher in the regenerated than in the "first" tips. This incidence of abnormality is significantly higher than that found in the controls.

6. The types of mosaics found included: hypodiploid/diploid/hyperdiploid; diploid/hyperdiploid; diploid/triploid; diploid/triploid/tetraploid; diploid/triploid/pentaploid; diploid/tetraploid.

7. A wide variety of abnormalities of mitosis was noted, including: "sticky" anaphases and telophases; anaphases and telophases with lagging or lost chromosomes or chromosome fragments; anaphases and telophases with bent spindles; multipolar spindles. Masses of relatively uncondensed chromatin and interphase nuclei with persistent connections between the daughter components were also present.

8. The evidence indicates that *T. torosus* larvae exposed early in development to low temperatures may suffer quite radical changes in chromosome number in some body cells, as well as less extreme abnormalities of mitosis. This is in accord with the findings of other workers for other species, and offers a clue to the possible mode of origin of the cytological abnormalities observed in *T. torosus* embryos subjected to shipment in an ice-and-water mixture.

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CARBONIC ANHYDRASE AND GROWTH IN THE OYSTER AND BUSYCON[1,2]

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The enzyme carbonic anhydrase is present in the shell-forming mantle tissue of a large number of mollusks (van Goor, 1948; Freeman and Wilbur, 1948). During the course of an investigation of the possible importance of this enzyme in the deposition of carbonate in shell formation it was noted that the carbonic anhydrase concentration of the conch *Busycon carica* was considerably less in larvae just prior to shell formation than in those which had recently acquired a shell. This suggested that the enzyme content may increase with development and raised the question as to the constancy of the enzyme content of the mantle tissue with growth of the adult. These aspects of carbonic anhydrase activity have been examined in the present study. The alteration of enzyme activity during growth has been investigated on the whole larvae of *Busycon carica*. For a study of enzyme activity during growth of adult mantle the oyster *Ostrea virginica* has been used. The results indicate that the carbonic anhydrase activity varies in a regular manner in these forms both during development and with growth of the mantle of the adult.

Methods

Ostrea mantle tissue and whole Busycon larvae taken from egg cases were ground and usually frozen. The tissue was then extracted in 3 to 9 times its weight of distilled water for two or more hours. Oyster sperm and eggs were washed in sea water by centrifugation before extraction. Tissue samples to be compared for enzyme activity were prepared at the same time and received identical treatment. The extracted material was centrifuged briefly to remove tissue and shell fragments and the carbonic anhydrase activity of the supernate was measured colorimetrically using a veronal buffer (Wilbur and Anderson, 1948). Sulfanilamide, a powerful inhibitor of mammalian carbonic anhydrase (Mann and Keilin, 1940), was found to inhibit the enzyme in Busycon and Ostrea. Accordingly, an aliquot of an experimental sample to which sufficient sulfanilamide was added served as a very satisfactory control. By comparing the rate of enzyme action in the presence and absence of sulfanilamide the action of tissue substances which affect the pH and buffer capacity of the medium is cancelled. The enzyme activity was calculated

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[2] We wish to acknowledge the helpful suggestions of Dr. A. F. Chestnut and Dr. D. P. Costello.

[3] Atomic Energy Commission Fellow.

from rates of the catalyzed and uncatalyzed reaction and expressed in units following the method of Main and Locke (1941).

Unfortunately no single tissue unit will serve satisfactorily as a basis for comparison for all the tissues employed. For mantle tissue, wet weight and dry weight have been used. For whole Busycon larvae, both with and without shell, the enzyme activity is expressed as units per individual and also per unit volume as determined by centrifuging thoroughly macerated larvae at ca. 825 × g. for 5 minutes. The latter method was also used for Ostrea eggs and sperm.

Results

Busycon larvae

The eggs of Busycon are laid in a string of flattened, disk-like cases which commonly contain two to three dozen or more individuals. Since the formation of the string of cases continues for some period, one has available for study in a single

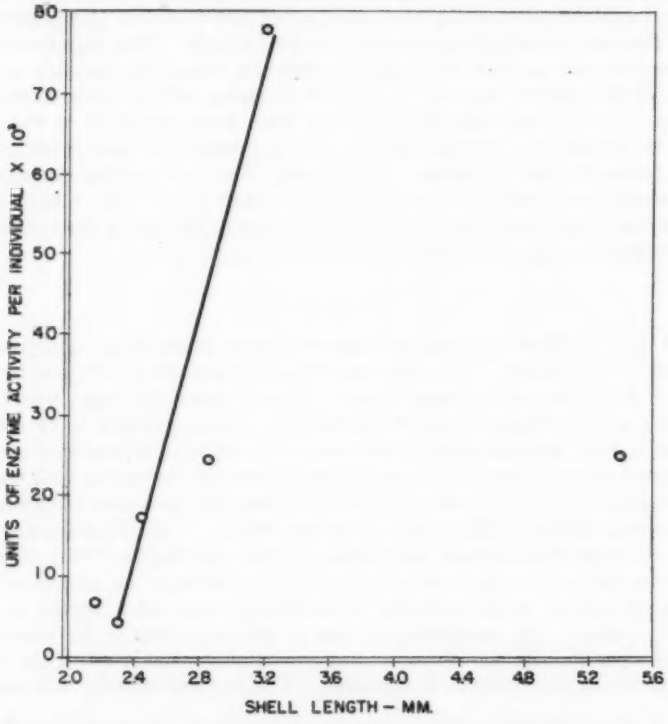

Figure 1. Carbonic anhydrase activity of Busycon larvae. Approximately 300 larvae of similar size from a single egg string were extracted for the determination of each point. Several egg strings were used. A similar relationship is obtained on plotting enzyme activity vs. unit volume.

string a series of developmental stages both with and without shell. The carbonic anhydrase activity of naked larvae and those with shell from such a string was measured on a sample of about 300 individuals of each type. The enzyme activity per individual was found to be 0.0049 units for those without shell and 0.23 units for those with shell, or an increase of 47 fold. The jelly within the egg cases was without enzyme activity.

The difference in enzyme activity in the naked and shelled stages suggested an examination of possible variation in enzyme activity with development in a series in which all larvae were depositing shell. Lots of 150 to 300 individuals in a desired size range were collected. A sample of 16 to 32 individuals was taken for an estimate of the average length, and the entire lot was then used for the measurement of enzyme activity. When the enzyme activity per individual is plotted against average length (Fig. 1) it is seen that the activity increases very markedly during the early stages of shell deposition. The activity of an individual 3.2 mm. in length may be nearly 20 times that of one 2.2 mm. in length. Clearly the increase per unit of tissue is also very great indeed. This may be shown by expressing the activity in terms of unit volume as determined by centrifuging macerated larvae, even though a large fraction of the total is shell. Whether enzyme activity falls off for individuals larger than 3.2 mm. (Fig. 1) or whether the value for the 5.4 mm. stage is aberrant is not known.

The changes in carbonic anhydrase activity with growth in Busycon reported here represent the enzyme activity of the whole larva. If the increase in enzyme activity found in Busycon is to be correlated specifically with shell deposition, the mantle tissue should also show the increase, since this tissue is responsible for shell formation. Unfortunately the measurement of enzyme activity of the mantle of individuals of 2 to 6 mm. in length will have to await the development of suitable micro methods.[4] The relation between size and enzyme activity of the adult mantle has, however, been investigated in Busycon and also in Ostrea.

Adult Busycon

Individuals ranging in length from 5 to 20 cm. and weighing from 8.9 to 587 gms. were used. Because of the difficulty of grinding mantle tissue, pieces of mantle were sliced very thin with a razor while frozen and extracted at low temperature for 48 hours. In a single series examined no consistent relationship between size and enzyme activity was found, although there was some slight suggestion that the mantles of the largest individuals had a somewhat lower enzyme content than the smallest individuals on a wet weight basis. Further study on this species would be desirable.

Adult Ostrea

In the adult oyster a definite relation was found between size and enzyme activity. The activity per unit dry weight or wet weight of mantle was in general inversely proportional to the length of the shell (Fig. 2). The activity of the smallest individuals may be several times that of the largest in any one collection.

The curves for oysters from two beds a few hundred yards apart are character-

[4] A micro method for carbonic anhydrase is being devised for such quantities of tissue.

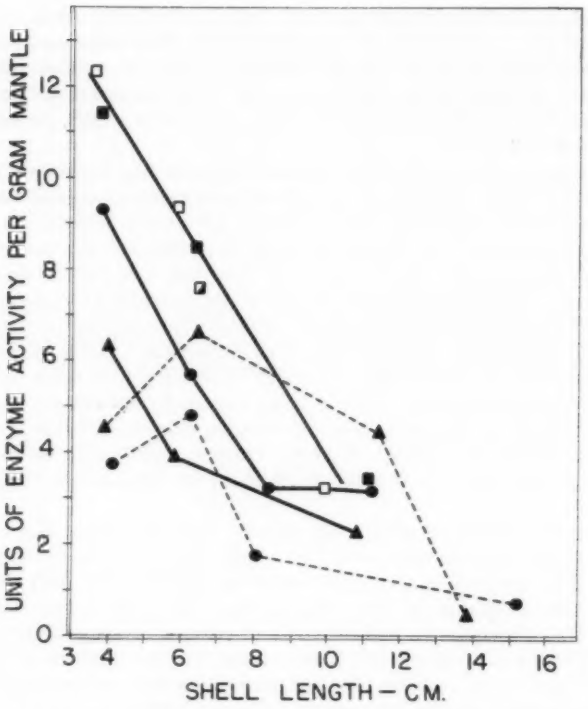

FIGURE 2. Carbonic anhydrase activity of oyster mantle. Enzyme activity is expressed per gm. wet weight. Dry weight gives a similar relationship. The shell length represents average measurements on the shorter shell. Data plotted with solid lines (———) are for oysters taken from a bed located on a sandy beach exposed to relatively rapid water movement. Data plotted with dashed lines (- - - -) are for oysters taken from an intertidal bed in muck containing fuel oil from ships and in a location not exposed to fast currents.

□ July 12, 1948.
■ July 12, 1948. Oysters kept in air 6½–7½ hours.
◪ July 12, 1948. Oysters kept in air 6½–7½ hours, then returned to water for 13 hours.
▲ October 31, 1948.
● March 5–6, 1949.

istically distinct both in their form and with respect to seasonal differences.[5] The data indicated in solid lines in Figure 2 were obtained from oysters collected between low and high tide levels from a sand beach exposed to relatively rapid water movement. Dashed curves (Fig. 2) are for oysters growing between tide levels in muck containing fuel oil from ships and in a location which was not subjected to fast currents.

[5] Dr. A. F. Chestnut of the Institute of Fisheries Research of the University of North Carolina, Morehead City, N. C., found active deposition of shell by oysters in this region throughout this period.

The enzyme activity of oysters exposed to air for 6½ to 7½ hours (Fig. 2, ■) was the same as that for oysters taken directly from sea water (Fig. 2, □).

By extrapolation of the curves in Figure 2 one might predict the size of oyster which would be completely deficient in carbonic anhydrase. Interestingly enough, oysters of this size have not been found in the beds from which the experimental material was collected.

Discussion

The presence of carbonic anhydrase in a wide variety of tissues of animals belonging to several phyla suggests that the enzyme plays a general role in the handling of metabolic carbon dioxide (van Goor, 1948). Such a role seems likely in mollusks as well, for the enzyme is present in several tissues. An analysis of washed eggs and sperm of the oyster showed that both gametes also contain carbonic anhydrase. The eggs had an activity of 3.6 units and the sperm 3.4 units per ml. of packed cells. Here too a general rather than a specific function appears probable. With differentiation the concentration of the enzyme may increase as described for Busycon larvae and has also been found in vertebrate tissues (Van Goor, 1948). Whether the marked increase in carbonic anhydrase bears a general relation to development and differentiation or has a more specific function in the rapid deposition of shell which occurs at this time, is not known.

In contrast to Busycon larvae, enzyme activity of the adult oyster mantle was found to decrease with increasing size. The possible physiological significance of this relationship for shell deposition obviously depends upon whether the enzyme activity becomes a limiting factor in the processes concerned with shell formation. If the enzyme is in excess, as it is in mammalian erythrocytes, a reduction may be of no consequence. An evaluation of the part played by carbonic anhydrase is being approached by studying the influence of enzyme inhibitors on the rate of shell growth as indicated by the deposition of radioisotopes.

The change in carbonic anhydrase content of the oyster mantle with age does not in itself reflect a decreased ability to synthesize the enzyme but may be due to a relative increase in cells or material not directly involved with carbonic anhydrase. Speculation in this regard appears unprofitable until such time as a histochemical technique for the enzyme can be applied to the mantle tissue.

The variation of carbonic anhydrase activity of the oyster mantle with size, habitat, and with the season suggests that these factors should be considered in comparing carbonic anhydrase activity of species of mollusks and perhaps other invertebrates.

Summary

1. The carbonic anhydrase activity of *Busycon carica* was found to increase markedly during development in the egg case. The enzyme activity of whole larvae during the early stages of shell formation may be more than 40 times that of larvae just prior to shell deposition. Larvae with shell may show an increase of nearly 20 fold in enzyme activity in growing from a length of 2.2 mm. to 3.2 mm.

2. In the mantle of the adult oyster *Ostrea virginica* the carbonic anhydrase activity was in general inversely proportional to the length of the shell. The activity per unit weight of mantle of small oysters may be several times that of large

oysters growing in the same location. Curves relating enzyme activity of the mantle to the size of the oyster were distinctly different for oysters growing in different habitats.

3. Carbonic anhydrase is present in both gametes of the oyster, the concentration per unit volume being of the same order in both.

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A PRELIMINARY HISTOLOGICAL STUDY OF THE INSEMINATION REACTION IN DROSOPHILA GIBBEROSA[1]

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The insemination reaction was first described by Patterson (1946) in Drosophila, and has been subsequently studied in numerous species of the genus by Wheeler (1947). The reaction occurs strongly, weakly or not at all depending on the species being studied. When present as a strong reaction (as in *Drosophila gibberosa*) it consists of a swelling of the vagina to three to four times the normal size following mating. This swelling is brought about by the secretion of fluid from the vaginal epithelium in response to some stimulus supplied by the semen; in at least some respects it resembles an immunological reaction. Commonly, especially in reactions resulting from the mating of different species, a more or less opaque mass develops in the lumen of the vagina; this is termed the "reaction mass."

Previous work has been limited to the observation of gross dissections and the determination of genetical results. The present study was initiated to extend the study to histological structure and to determine the specificity of the reaction, and perhaps the steps involved, by the artificial insemination of materials other than semen.

Materials and Methods

Drosophila gibberosa was selected because of its large size, availability and ready rearing on standard media. Since adults of this species do not mate until nine to fourteen days after emergence, males and females were kept separately in humid milk bottles and fed bananas or a yeast-honey mixture during this period.

Mating can be obtained with single females and several males in separate containers, but for the purposes of this work mass methods were more satisfactory. Ten to twenty females and a similar number of males were placed in glass containers of approximately one pint capacity. The containers were lined with wet filter paper to maintain a high humidity. Reasonable numbers of matings occurred within 30 minutes. Mating pairs were isolated by inverting small vials over them, the vials being numbered corresponding to notes kept on each pair.

The abdomens of mated females were cut off and fixed in Brazil's (alcoholic Bouin's) fluid at 0, 5, 15, 30, 60, 120, 180, 240, 300, and 360 minutes after the beginning of mating. In some tests intervals as long as 11 hours were used. They

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were then washed, dehydrated, sectioned in paraffin (Tissuemat), and stained with Delafield's haematoxylin and eosin or acid fuchsin and congo red by routine methods.

For artificial insemination the desired organs were dissected free in saline,[3] cleaned of adhering tracheae and other tissues, and ground into a brei in saline using a small mortar and pestle. Capillary glass tubes sealed onto ordinary syringe needles were drawn to desired fineness and used for injecting into the vaginae. Etherized females were placed ventral side up on the stage of a binocular microscope, held gently with forceps, and the needle (preferably held in an adjustable mechanical stage) manipulated through the gonopore into the vagina. In the present preliminary work, the injections were not quantitative but were roughly the same volume. Artificially inseminated females and ones injected with non-semen experimental fluids were prepared for histological examination in the same manner as the normally mated females.

Fluids injected into the lumen of the vagina artificially include:

1. Yeager's cockroach saline;[3] this has a tonicity equal to a 1.3 per cent solution of NaCl.
2. 2 per cent NaCl solution.
3. 2 per cent gelatine in cockroach saline solution.
4. brei of testes in saline solution.
5. brei of male accessory glands in saline solution.
6. brei of male accessory glands plus testes in saline solution.
7. brei of entire male fly in saline solution.

The volume of epidermal cells of the vagina was estimated by making camera lucida drawings of each section on calibrated graph paper, adding up the number of squares occupied by the drawings (estimating partial ones), and multiplying by the thickness of the sections. Probably a rather high percentage of error is involved in these estimates, but since the differences to be discussed are differences of several magnitudes the errors would not seem serious. It should be remembered, however, that all areas and volumes represent measurements on sectioned material; the figures are doubtless smaller than life size but should be accurate for relative sizes.

Results

1. Macroscopic features of vaginae of virgin females: The term vagina in insects has been variously used. Snodgrass (1935) regarded the vagina as that part of the female reproductive tract which extends posteriorly from the base of the median oviduct to the ovipositor. In dipterous insects, Pantel (1910) referred to it as the uterus (Townsend, 1911). Nonidez (1920) divided this region into two parts, a uterus proper and a vagina. For Drosophila, Patterson (1947) followed the terminology of Snodgrass but added the term "vaginal pouch" for the portion of vagina which extends antero-ventrally. During the course of insemination reaction, the wall of the vaginal pouch is capable of great distention, the wall of the vagina remains unchanged. For descriptive convenience, the terminology of Patterson was adopted in the present study.

[3] Yeager's cockroach saline: NaCl, 10.93 grams; KCl, 1.57 grams; $CaCl_2$, 0.85 gram; $MgCl_2$, 0.17 gram; $NaHCO_3$, 0.17 gram per liter of double distilled water.

In *Drosophila gibberosa* the vagina is a whitish pear-shaped body, situated at the posterior end of the abdominal cavity just beneath the rectum. The vaginal pouch is about one-third as large as the vagina and is situated antero-ventrally to the origin of the parovariae and ventral receptacles. Both vagina and vaginal pouch are provided with a well-developed muscular wall and are without significant demarcation externally. The gonopore is enclosed between two valves of the ovipositor which are located at the posterior end of the abdomen externally.

2. Microscopic structure of vaginae of virgin females: The vaginal wall is composed of three distinct layers. The innermost layer is a transparent thin non-cellular cuticular intima which is more prominent in the vagina than in the vaginal pouch. Apparently it was the endocuticular portion of this that was called a separate fibrous layer by Nonidez (1920) in *Drosophila melanogaster.* Second, there is a single layer of cuboidal epithelial cells, the nuclei of which are more or less oval, located usually in the middle of the cells and generally with distinct chromatin threads and one or two nuceoli. The epithelial layer is much thicker than the intima: it is slightly thinner on the wall of vaginal pouch and with more scattered and sparse cells as compared with aggregated and dense ones of the vagina. The epithelium and the intima are thrown deeply into many folds within the lumen, especially so on the wall of the vaginal pouch; thus the layers of cells appear to be superimposed on others in the section. Third, externally the vagina is lined by a thick muscular layer which is much thinner and sparser on the wall of the vaginal pouch. Presumably a basement membrane and some other slight connective tissue elements are present but these are not sharply demarcated. The volume of epithelium measured from three sets of serial sections averages 1,270,000 cubic microns.

3. Macroscopic features of vaginae of mated females: Immediately after mating the vaginal pouch begins to increase in size. In some cases the vaginal pouch reaches its greatest distention about two and one half hours after mating; in other cases four hours is required to attain the greatest distention. The size at greatest distention is about four or five times as large as the normal one. The actual size attained is the sum of (1) initial size, (2) amount of semen, (3) amount of material added to semen, and (4) any changes that occur in the vaginal wall. These will be discussed later.

The wall of the vaginal pouch becomes very thin and transparent. The vagina only slightly increases in size. Within the vaginal pouch an opaque reaction mass can be seen under the microscope. The vaginal pouch begins to contract after reaching its greatest distention and the opaque reaction mass also gradually decreases in size. It returns to original size and form about five hours after mating.

4. Histology of vaginae of mated females: As the wall of vaginal pouch is gradually distended after mating, its epithelial cells become flatter and flatter and the folds within the lumen finally disappear. The intima also becomes thinner. The muscular layer becomes very thin and the muscles widely separated. The vagina is less distended; its epithelial cells remain cuboidal. As a rule the nuclei of epithelium of reacting vaginae are generally more dense as compared with those of the virgin female and without the diffuse appearance of chromatin threads; the nuclei are frequently elongated and generally located at the base of the cells. The cell volume increases to about four times the normal at its greatest distention; it averages 4,800,000 cubic microns from the measurement of three sets of serial sections of vaginae.

After reaching its greatest distention, the vagina gradually returns to its normal histological structure and about five hours after mating there is no significant histological difference from that of the virgin female. The reaction mass is composed of spermatozoa and substance of acidophilic staining property; its increase and decrease of size keeps well in pace with the distention and contraction of the vagina. The entering of spermatozoa into the ventral receptacle can be traced in the sections.

5. Macroscopic features of vaginae of artificially inseminated females: Testes brei in saline, male accessory gland brei in saline, testes plus male accessory gland brei in saline, entire male fly brei in saline, cockroach saline, 2 per cent NaCl and 2 per cent gelatin in saline were used for artificial insemination. The results are different for different injection materials.

After the injection of testes brei the vaginae undergo macroscopic changes similar to those of the normal mated female. The same feature is found in the vaginae of testes plus male accessory gland injections but the distention is of lesser degree. Injection of saline also brings about the same amount of distention. The injection of male accessory gland brei, entire male fly brei, 2 per cent gelatin and 2 per cent NaCl may bring about an unsignificant increase of vaginal size by mechanical distention of fluid injected into the lumen, but the vaginal pouch does not thin out as in other experiments.

6. Histology of vaginae of artificially inseminated females: The vagina of testes brei injections undergoes a histological change similar to that of the mated female as far as the epithelium, intima, muscles and reaction mass are concerned. The volume of epithelial cells also increases; an average of measurement of two vaginae has the same figure as that of the mated female, that is 4,800,000 cubic microns.

The vagina of testes plus male accessory gland brei injections undergoes a similar histological change to that of the previous case, but the cells on the wall of the vaginal pouch do not thin out so extensively. The reaction mass is somewhat smaller in size, but the significance of this is open to some question, as will be discussed later.

The vaginae of male accessory gland brei injection, whole male brei injection and 2 per cent gelatin injection did not show any significant histological change.

The vaginae injected with saline (which apparently is hypotonic) undergo a peculiar histological change, in which the vaginal pouches thin out moderately with the outer muscle layer retained. Unlike the nuclei of the vaginal epithelium of the mated female, the nuclei are generally of diffuse appearance. Th cytoplasm is generally more lightly stained by eosin as compared with that of the virgin or mated female. Although the vaginae are distended moderately there is no reaction mass found in the lumen. The volume of cells also increases, but to a lesser degree, average of measurement of two sets of serial sections is 3,420,000 cubic microns.

Contrary to the results from saline injection, the vaginae after injection of 2 per cent NaCl does not exhibit significant distention. The cytoplasm of the vaginal epithelium is generally heavily stained by eosin and significant shrinkage is shown at certain areas. The nuclei are also slightly shrunken. The cell volume does not increase, an average of measurement of two sets of serial sections is 1,311,000 cubic microns which is close to the figure of virgin female.

The reaction of vaginae to different kinds of inseminations is summarized as follows:

Kinds of insemination	Increase of vaginal size	Increase of volume of vaginal epithelium	Reaction mass plus intense staining
Normal mating	++	++	+
Testes in saline	++	++	+
Testes plus accessory glands in saline	++	++	+
Saline (hypotonic?)	+	+	−
Male accessory glands in saline	+	0	0
Entire male fly in saline	+	0	0
2% gelatin in saline	+	0	0
2% NaCl (hypertonic?)	+	0	0

The volume of epithelial cells of vaginae of virgin females, mated females and females artificially inseminated with testes brei, saline and 2 per cent NaCl were measured. As stated under the MATERIALS AND METHODS, these figures are relative rather than absolute because they were made on sectioned material which unquestionably shrunk somewhat during the course of preparation. The average figures for the volume of cells are tabulated as follows:

Type of female	The volume of vaginal epithelium (in cubic microns)	Average (in cubic microns)
Virgin female	1,568,116	1,270,000
	1,285,274	
	1,046,470	
Mated female	4,056,381	4,800,000
	4,895,334	
	5,448,870	
Female of testes injection	3,502,842	4,800,000
	6,097,545	
Female of saline injection	3,433,653	3,420,000
	3,596,533	
	3,234,726	
Female of 2% NaCl injection	1,407,400	1,311,000
	1,215,358	

DISCUSSION

Patterson (1946) described the presence or absence of the insemination reaction in thirty-five species of Drosophila. In 1947 he made an experiment of homogamic matings of eight species and twenty-six crosses of the species of the genus Drosophila. He found that in homogamic matings it takes five to ten minutes after mating for the sperm to move forward to the point of origin of the ventral receptacle and several hours to enter the ventral receptacle. In heterogamic matings similar movement of sperm take about forty-five minutes and the spermatozoa frequently form a tangled mass about the opening to the ventral receptacle. He pointed out that this has the effect of retarding or inhibiting the entrance of sperm into the lumen of the receptacle. In many interspecific crosses, the sperm never fertilize the eggs due to the deleterious effect of the insemination reaction and the resulting reaction mass. The egg may disintegrate in the cavity of the vagina.

Patterson (1946) inferred that the insemination reaction may have three possible functions: It may have the effect of (1) preparing the reproductive tract for the fertilization mechanism, especially in the case of homogamic matings: and it may have a direct bearing on the problem of speciation both (2) by deterring genic interchange and (3) by limiting the number of matings an individual female may have.

As regards the induction of an insemination reaction among homogamic matings of *Drosophila aldrichi*, Patterson (1947) found in one case that the vagina had already reached its maximum reaction, although the female was dissected before the termination of mating. Yet, in the distended vagina or ventral receptacle there was not a single spermatozoon. From this, he assumed that the spermatozoa are not the active agent to bring forth the insemination reaction. He undertook a convincing experiment to support his assumption. In the backcross of the sterile F_1 male from the *mulleri/mojavensis* with *mulleri* females, the vagina is slightly distended. Since the semen of this F_1 hybrid male is sperm free, the result demonstrates conclusively that the presence of spermatozoa are not necessary for the induction of the insemination reaction.

The knowledge about the exact composition of the semen of Drosophila is meager. According to Nonidez (1920) the accessory glands secrete a sticky fluid with floating refractive granules and this fluid becomes mixed with the spermatozoa from the testes to form the ejaculate. However, although the spermatozoa were found not necessary to induce reaction, at least in the above experiment, Patterson does not conclude that the secretion of the male accessory gland has such a function. He stated that the semen of F_1 hybrid of *mulleri/mojavensis* is sperm-free but does not exclude the possibility that degenerative products from the abnormal testes may constitute a part of the semen. This must await fractionation of semen into its components. Patterson points out superficial similarities to immunochemical reactions but direct evidence is lacking.

Wheeler (1947) classified seventy-eight species belonging to the genera Chymomyza, Scaptomyza and Drosophila of the family *Drosophilidae* into three classes according to the degree of insemination reaction in homogamic matings. Class 1 is composed of those forms in which no reaction is apparent; he lists seventeen species including the common *D. melanogaster*. Class 2 contains those in which a slight or moderate reaction develops; he lists forty-one species. Class 3 contains those in which there is a considerable enlargement of the vagina and formation of a dense opaque reaction mass; he lists twenty species. He also considered the problem of evolution of Drosophila from the viewpoint of insemination and the possible function of an insemination reaction in homogamic matings. As indicated by Patterson, it may have the effect of preparing the reproductive tract for the fertilization mechanism in homogamic matings. It may be correlated with the slower movement of the spermatozoa into the ventral receptacle of the immobilization of the sperm possibly due to the greatly increased viscosity of the reaction mass.

In the present study the size of the vagina was found to increase in various degrees after natural and certain artificial inseminations. The increase of size at the maximum reaction was greater for natural insemination and testes brei injections than for the other materials. The reaction mass is present in the vagina of natural insemination, testes brei injection, testes plus male accessory gland brei injection. The vaginae which increased in size significantly also showed an increase of the

volume of epithelial cells, but degree of increase is not the same in all cases. The vaginae of natural insemination and testes brei undergo the maximum increase of cell volume, a lesser degree was found in the testes plus accessory gland brei injections and a still lesser degree in the saline injections.

At first sight it may seem surprising that the vaginal epithelial cells increase in volume, especially since distention of the vagina makes the vaginal wall become thinner. But it should be remembered that the vaginal wall is only an invaginated part of the general body integument, and that it is well known that the epidermis of arthropods increases greatly (common range 2–5 times in thickness) during the period when a new cuticle is being deposited (Wigglesworth, 1939). The reaction of the vaginal wall, then, seems quite in keeping with reactions to be expected of arthropod epidermal cells.

The vaginae with a reaction mass have their epithelial nuclei somewhat dense in appearance. In other cases, even though the vagina may increase significantly in size, as in saline injections, the reaction mass is absent, the nuclei generally show diffusely oriented chromatin threads as those of the virgin female, and the cytoplasm is more lightly stained.

In mated females the vagina increases in size after mating (Patterson) and therefore the reaction mass must contain both semen and material added to it. Presumably the material added to the semen is secreted by the vaginal epithelium but origin from associated organs is not excluded. In addition the cells of the vaginal epithelium increase in size and stainability; therefore they too have material added to them. In response to the stimulus from the semen, these cells must absorb material from the blood (and perhaps alter this metabolicly). If these cells secrete the material into the lumen of the vagina, then they must absorb from the blood enough to account for the increase in both the reaction mass and in the cells. Obviously there is a strong reaction by the cells to the stimulus afforded by the presence of semen (or some component in the semen).

The reaction brought about by mating and by testes brei or testes plus male accessory gland brei injection reveals all the features just mentioned. However, in the specimens injected with saline, although the size of the vagina and the volume of epithelial cells increase, the nuclei are loose and the cytoplasm is lightly stained and the reaction mass is never present. From the evidence that the cytoplasm is lightly stained it is assumed that increase of cell volume is simply the result of the uptake of water from the injected saline on account of the lower osmotic pressure of the saline used as compared with the protoplasm of the epithelium. In order to make a more accurate comparison of the staining susceptibility of cytoplasm of vaginae after various treatments, sections of vaginae from a mated female, a female of saline injection and a virgin female were arranged on the same slide so as to obtain a uniform staining process. The results show that the cytoplasm of vaginal epithelium from the mated female is more heavily stained by hematoxylin and eosin than that from the female of saline injection, while that of the virgin female has a staining susceptibility intermediate between those two. Obviously, the cytoplasm of vaginae injected with saline is diluted and the dilution is almost certainly due to the uptake of water from the saline solution injected. The contraction of vaginal epithelial cells by injection of 2 per cent NaCl contributes further support to this

assumption, because the osmotic pressure of 2 per cent NaCl is generally higher than that of the protoplasm of insects.

Since the injection of male accessory glands did not induce any insemination reaction in the present data, it is assumed that the secretion of the male accessory gland is not the substance to initiate the reaction. Quantitative effects are not yet precluded but it seems most reasonable to conclude tentatively that the reaction is due to some semen component derived from the testes. As Patterson presented some evidence that the spermatozoa are not the active agent to induce reaction, the inducing substance may be a factor in the testes besides spermatozoa. The active component is not yet known, and we have only two clues about it: (1) it is probably from the testes, and (2) its action suggests protein.

Summarizing the material presented in this discussion: The insemination reaction can be broken down into five component parts: (1) an increase in size of vagina, (2) an increase in volume of epithelial cells, (3) the presence of a reaction mass, (4) a dense appearance of nuclei, and (5) heavily stained cytoplasm. To a certain extent these are experimentally separable. All are produced by mating and by the injection of brei containing testes. (1) plus (2) can be produced by hypotonic saline solution; (4) plus (5) can be produced by hypertonic saline solution; (1) alone is produced by osmotically inactive solutions. It would seem likely that (3) alone could not be produced, *i.e.*, that the reaction mass necessitates the other component steps, but this cannot be proved until the active agent is available for artificial insemination.

Summary

1. Macroscopic features and microscopic structure of vaginae of *Drosophila gibberosa* Patterson and Mainland are described in virgin females, mated females, females with testes injection, male accessory gland injection, testes plus male accessory gland injection, whole male brei injection, 2 per cent gelatin injection, saline injection and 2 per cent NaCl injection.

2. The wall of the vagina consists of intima or cuticle, epithelium and muscle layers.

3. The insemination reaction occurs in vaginae of mated females, females with testes injections, and testes plus male accessory glands injections. The reaction consists of five characteristic features: (a) increase of size of vagina, (b) increase of volume of epithelial cells, (c) presence of reaction mass, (d) dense appearance of nuclei, and (e) more heavily stained cytoplasm. Some but not all of these features have been produced separately by artificial insemination with experimental solutions.

4. The increase in volume of the vaginal epithelial cells is compared with the known increase in volume of epidermal cells during deposition of a new cuticle.

5. Cell volumes can be altered by the injection of hypertonic and hypotonic saline solutions as shown by both volume and intensity of staining.

6. The reaction of vaginae of mated females reaches its maximal distention two and a half to four hours after mating. Then it decreases in size gradually and returns to its normal size five or six hours after mating.

7. The reaction mass within the vagina is composed of spermatozoa and substances of acidophilic staining property. The increase and decrease of the size of

the reaction mass during the course of the reaction keeps well in pace with the size of the vagina.

8. The insemination reaction of vagina as demonstrated by Patterson (1947) seems not induced by spermatozoa, and the present data show that it is not induced by male accessory glands either. Since the semen of Drosophila consists of secretion of accessory glands, spermatozoa and some substances from testes besides spermatozoa, it is suggested that the agent which induced the insemination reaction is most likely some factor from the testes other than the spermatozoa.

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NOTE ON THE GROWTH OF ARMS IN THE PLUTEUS OF ARBACIA PUNCTULATA

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I

Allometric studies have dealt extensively with invertebrates whereas descriptions of growth in which time is explicit rest largely on measurements of prenatal and postnatal vertebrates. In logarithmic form the allometric equation $y = bx^a$ predicts a straight line (Huxley, 1932). Glaser's (1938) linear $\log w = k \log (2T + 1) + C$ as Kavanagh and Richards (1942) have shown is essentially a variant of the allometric equation in which weight, w, can be equated with y; the time factor with x, while $k + C \log 2 = \log b$. Both types of analysis have been subjected to rigorous criticism. Thus Kavanagh and Richards (p. 168) point to "varying degrees of concavity or convexity, including occasional approximations to linearity." Among the case of "upward concavity" they cite Figure 11 (Glaser, l.c., p. 41) which happens to illustrate the nutritive disturbances that characterize the weaning period in mice. However, there are also "a few examples of linearity." Those specifically cited by Kavanagh and Richards refer to the growth of fresh weight in the chick; to the growth of embryonic chick brain; and to the accumulation of such chemical entities as arginine, cystine, and purine. Many of the data dealt with by Glaser are subject to considerable experimental errors and no doubt their treatment could be improved, yet even so it is permissible to ask whether cases where a single stanza of growth is admittedly linear are not as significant as those to which objection is made. This query is especially pertinent since the two stanza treatments result in correct orders of magnitude for the allantoic removal of Ca from the shell (Glaser, p. 27), and also enable us to account within 247 mgs. for the total protein metabolism of the developing egg (Glaser, p. 31).

II

We now turn to E. B. Harvey's (1949) measurements on the growth of the long anal arm of the *Arbacia pluteus*. Here we have an adequately timed invertebrate material. Like all the others, this arm is a definite organic entity whose lengths we may consider proportional to a series of weights (themselves controlled by increases in the lengths of skeletal rods) somewhat diversified in structural detail but probably uniform in chemical composition. If valid, increasing arm lengths would involve proportionately increasing amounts of essentially the same organic categories. How closely then does the growth of this structure follow the linear law?

Harvey's cultures were fed on diatoms. However, even though derived from the same batch of eggs, individual plutei and their arm lengths (measured in

microns) proved quite variable. The data therefore are averages based on selection of the better developed specimens. Since nutrition markedly affects arm lengths, especially in plutei older than 4 days (Harvey, l.c., table, p. 294) we can assume that selection influences the uniformity of the measured material by segregating comparable samples of the best fed individuals.

Developmental time, recorded in days, weeks, or months, and now reduced to days, does not include pre-pluteal time. This introduces an error in timing, but the error is reasonably constant and if corrected, would only improve the distribution of the points. These are plotted in Figure 1 according to the modified allometric equation.

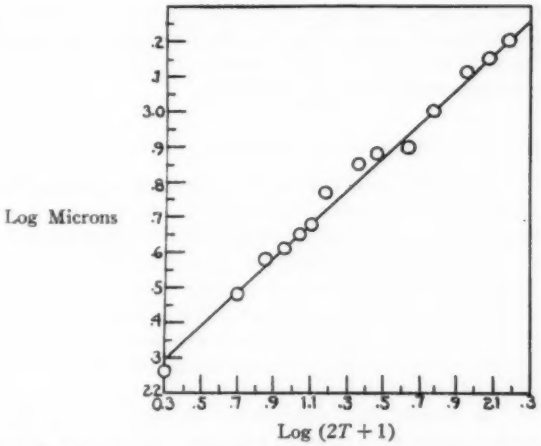

FIGURE 1. Modified allometric plot of growth in length of anal arms in the pluteus of *Arbacia punctulata*. Original data of Ethel Browne Harvey.

As the figure shows, arm lengths for the first and twenty-first days are less than the equation demands while those of the 7th, 11th and 14th days are greater. Since the 14th and 21st days fluctuate about the line, we can attribute these two deviations to chance. Whether the 7th and 11th days represent systematic deviations may be an open question. Certainly the remaining points closely approximate the predicted straight line. It would be curious if this approximation were attributable to the accidents of a selection involving judgement and made for other purposes. Thus in spite of criticisms some of which may be valid, the modified allometric equation seems to apply to a wide range of growth measurements.

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REGENERATION IN AN EARTHWORM, EISENIA FOETIDA (SAVIGNY) 1826. II. POSTERIOR REGENERATION

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This contribution is the second in a series presenting the results of an attempt to obtain, for one particular species of earthworm, complete characterization of regenerative capacity with reference to exact levels. In this part, posterior regeneration, and by anterior substrates only, is considered.

Summary of Previous Work

The information hitherto available as to posterior regeneration by anterior substrates of *E. foetida,* is summarized in Table II. Morgan once referred to one posterior regenerate, a monstrosity, as "a double tail." Other posterior regenerates of various workers from Morgan (1895) to Moment (1946) were not definitely characterized as to morphological nature. Even after rediscovery of anterior heteromorphosis in 1898–99 (see part I) no possibility of posterior heteromorphosis seems to have been anticipated. Very little evidence is available that might indicate morphological nature of many regenerates and the most useful clue, number of segments, may be of little value in certain circumstances.

The uncertainty as to nature of regenerates, paucity of data at most of those levels where operations were made, and lack of data for so many other levels, indicated need for a systematic investigation of regeneration at each level.

Material, Methods and Nomenclature

In addition to the information in corresponding sections of part I, only a few comments are necessary.

Especial effort was made to avoid use of animals that had undergone amputation followed by enteroparietal healing without regeneration (see Gates, 1949b).

Previous work on other species had shown that accumulation of ingested material, such as earth or manure, which could not be passed out by the operated animal, frequently resulted in rupture. Regenerates of survivors were always deformed. To obviate those abnormalities and the higher mortality, as well as effects of pressure of accumulations on the regenerating region of worms which did not rupture, operated animals were at first kept in filter paper or paper towelling. Fibres of such materials were, however, ingested by some individuals in amounts sufficient to result in rupture. This always proved fatal, in *E. foetida,* within 48 hours. A fine grade of cheesecloth was then used. Edges of the cloth had to be hemmed so that free ends of threads were not exposed, as experience with that material, as well as with coarse towelling, showed that worms were able to pull

TABLE II

Posterior regeneration in Eisenia foetida

Level	Number of regenerates				Number of segments in regenerates	Comments on substrate	Comments on regenerate	Time days*	Author	Date	Page
	H	U	T	O							
3/4				5				−6	Michel	1898	261
4/5				5				−6	Michel	1898	261
5/6				5				−6	Michel	1898	261
6/7	(1?)				+			?	Korschelt	1898	79
7/8				5				−6	Michel	1898	261
	(1?)				+			?	Korschelt	1898	79
8/9				5				−6	Michel	1898	261
	(1?)				+			?	Korschelt	1898	79
9/10	(1?)				(7)	6s+"3 angeschnittenen segmenten" See note		?	Korschelt	1898	80
10/11		1			0	(a)	Bud	20	Korschelt	1897	100
11/12				5				−6	Michel	1898	261
12/13				5				30	Morgan	1895	458
				5				−6	Michel	1898	261
13/14											
12/13+?	(1?)				4	12+"einigen verletzten segmenten"		?	Korschelt	1897	100
14/15				5				40	Morgan	1895	458
15/16				15				60	Morgan	1897	571
16/17				4				?	Morgan	1895	458
				1				7	Hescheler	1896	234
17/18				1				7	Hescheler	1896	234
				5				−6	Michel	1898	261
18/19				1				7	Hescheler	1896	234
19/20				3				40	Morgan	1895	458
				1				7	Hescheler	1896	234
		1					"Knob"	60	Morgan	1906	469
20/21				1				7	Hescheler	1896	234
			3	22	28? ? ?		See note (b) "long posterior ends"	110	Morgan	1897	571
				27				49	Liebmann	1943	600–1
21/22			1		15			?	Korschelt	1898	80
			1		22 "abn"			60	Morgan	1906	465
22/23				3			?	120	Morgan	1895	458

TABLE II—*Continued*

Level	Number of regenerates				Number of segments in regenerates	Comments on substrate	Comments on regenerate	Time days*	Author	Date	Page
	H	U	T	O							
23/24				1				60	Morgan	1895	458
			2		"17–20 abn," 18			60	Morgan	1906	465–9
			1	5			"a half inch"	90	Morgan	1895	458
24/25		4	1	15		See notes (c) and (d).	"with double tail"	110	Morgan	1897	571
		1	2		3+, 12+, ca. 40			60	Morgan	1906	465
				1				10	Korschelt	1896	58
			1		50			60	Morgan	1906	465
				?		Mature		30	Liebmann	1942	378
25/26			?			Immature (age?) regenerated completely		?	Liebmann	1942	378
				6				?	Morgan	1895	458
26/27			2		23, ca. 30			60	Morgan	1906	465
27/28				1				30	Morgan	1895	458
28/29											
29/30											
30/31		maj.	min.		20–30 mm.		See note (e)	30	Liebmann	1943	602
31/32			1		25			60	Morgan	1906	463
32/33			1	2	72			90	Morgan	1906	463–73
33/34			3		13 (abn) 45, 54			60–90	Morgan	1906	463–73
34/35			3		30+52, 53	See note (f)		60–90	Morgan	1906	463–73
35/36			1		45			60	Morgan	1906	463
36/37			1		60			90	Morgan	1906	473
37/38			1		37			60	Morgan	1906	463
38/39			1		52+			30+	Morgan	1906	464
40/41			7		47–56	See note (g)	Mean = 52 S	30+?	Moment	1946	496
50/51			180		22–57		Mean = 39.5 S	30+?	Moment	1946	496
60/61			19		19–38		Mean = 29.6 S	30+?	Moment	1946	496
70/71			31		6–31		Mean = 21.3 S	30+?	Moment	1946	496
80/81			118		2–22		Mean = 9.8 S	30+?	Moment	1946	496
			5		6–7–9–15–19	See note (i)		26	Michel	1898	260
−L 20S					6–7–13–17–20		Same regenerates two weeks later	40	Michel	1898	260
−L 10S			4		5–5–6–7			26	Michel	1898	260
−L 12–10S			?		"a few segments"			?	Morgan	1906	471
−L 5S			5		3–4–4–5–6			26	Michel	1898	260

off and swallow lint in sufficient quantity to result in rupture of posterior end of substrate or the regenerate.

Use of starved or starving animals, in addition to reducing mortality, also provided greater uniformity in experimental conditions, for, if food was available, anterior substrates, having mouths, often did eat, while posterior substrates from the very same animals, having no mouths, could not eat.

Anterior substrate refers to any "cephalized" portion of the body, regardless of size, comprising the buccal region and extending back to a single posterior transection.

Summary of Results

Healing in some individuals was enteroparietal rather than cicatricial. Such specimens did not regenerate in the two or three months before experiments were

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Notes to Table II

H—Cephalic
U—Uncertain
T—Caudal
O—None
S—Segments

* Time may indicate day of death of last specimen, of last observations, or of termination of experiment. In case of Morgan's experiments time is approximate—because of infrequency and irregularity of examination of operated animals, and method of presentation of data.

(a) The cut was diagonal and from the data provided in the text as quoted above, level of regeneration, in accordance with previous practice (see Part I, Table I, note c), should be taken as 9/10. However, Figure 8 shows a substrate of at least 7, possibly 8, complete segments and major portions of two others (missing portions apparently not regenerated). Level of regeneration, according to the figure, should, perhaps, be taken as 10/11.

The figure does not have, in the seven-segment regenerate, the terminal notch that indicates, in certain other regenerates on the same page, presence of an anus.

In *Regeneration and Transplantation* (1907, p. 80, fig. 50B) the same substrate appears to have been reproduced but the regenerate, again without a terminal notch, is marked off into eight divisions, the last of which could represent a segment or a prostomium. Alongside (in Fig. 50A) is shown a substrate of 11 presumably complete segments with a regenerate of nine segments of which the terminal is notched (1927, p. 332, Fig. 188). If 50A is a representation of an actual specimen, caudal regeneration anterior to 20/21 is indicated.

(b) Figure C (Morgan, 1901, p. 8), shows a 20-segment substrate with a regenerate of 28 segments. Although 20/21 is the anteriormost level of regeneration definitely recorded by Morgan, two statements seem to indicate regeneration further forward.—"Anterior ends containing from thirteen (?) to thirty segments sometimes regenerate posteriorly" (summary, 1897, p. 584), and "as I have shown elsewhere, the power of regenerating a posterior end ceases rather suddenly about the level of the 15th segment" (1906, p. 463). In both, reference presumably was to a doubtful case of regeneration (posterior) at 13/14 by a two-surfaced fragment and not an anterior substrate. Note also "no cases of survival of as few segments as fifteen were ever found" (1895, p. 458).

(c) Number of segments in substrate, according to text, 25 or 24, according to table (I, p. 571), 24. The latter is taken as correct as Figure 1 shows 24 segments in the substrate.

(d) Figure 1 (Morgan, 1897) shows a regenerate with numerous metameric abnormalities, bifurcated distally, one bifurcation represented as having an anus. This is the posterior regenerate that was morphologically characterized.

(e) "The majority die after regenerating short pieces" at least some of which may well have been cephalic. "Those which survive do reform full size individuals."

(f) At EL 34/35 eight regenerates: abn., 19 (abn), 31, 37, 36–40 (abn), 40 (abn), 55 (abn), 61. Time, *ca.* 60 days. Actual level of regeneration could have been from 31/32–38/39.

(g) Morgan (1906) experimented in two regions behind 40/41; at the "middle" of the body (EL 50/51) and after excision of L25–20S (EL 75/76–80/81). Actual levels, when deter-

terminated (but see note (h), Table II). In other specimens a small bud was formed. At certain anterior levels, the bud shortly ceased to grow. At more posterior levels, further growth often enabled recognition of a growth zone producing segments by the rapid ventral or terminal methods (see Gates, 1948). In the former case, the ventral method eventually gave way to the terminal and finally the growth zone was reduced to a very small anal region, as in normal worms. Thereafter new segments were formed by the slow terminal method of normal, late-juvenile or post-juvenile growth. Regardless of length of time animals were kept after operation the regenerate always remained recognizable as such and clearly distinguishable from substrate.

Posterior regeneration, at levels behind 40/41, was obtained throughout the entire year.

A. After a single cut

Anterior substrates of 15 or fewer segments did not regenerate and in many cases probably did not live long enough to do so. At levels 16/17–19/20 small buds were formed but soon ceased to grow. These buds were still unpigmented, metamerically undifferentiated and without terminal sculpturing 80–90 days after operation when experiments had to be terminated. The external appearance of such regenerates was more like that of a cephalic than a caudal bud.

Regenerates (metamerically organized growths) were obtained at each intersegmental level from 20/21 posteriorly. In the Cambridge experiments percentage of regeneration gradually increased from 20/21, with 100% regeneration on substrates having transections at levels 32/33–39/40. With but two exceptions, all regenerates were caudal.

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mined, were at 41/42–64/65 and 64/65–88/89. "In order to compare the rate of regeneration at the beginning and toward the end of its period of growth some of the same lot of worms that gave the records of Table IV were kept alive for another month (Jan. 6 to April 6) in order to see how much further the regeneration of new segments would continue. The results are given in the following Table IX" (1906, p. 473). But the worms of Table IX were "Cut behind girdle" as proved by number of segments, "at middle" and near posterior end, while none of the worms in Table IV were cut near the posterior end nor behind the girdle. Accordingly the comparable results were those of the two sets of regenerates at EL 50/51. Omitting from consideration those substrates in which segment number was not determined, the results appear to be as follows.—Two months; four substrates of 41, 47, 55, and 56 segments with regenerates respectively of 58, 44, 43, and 39 segments, average number of segments 46. Three months; seven substrates of 41, 48, 51, 51, 52, 53, and 59 segments with regenerates respectively of 53, 52, 46, 40, 36, 42, and 32 segments, average number of segments 43.

(h) Morgan's Table I (1897, p. 571) seems to indicate that regeneration at levels 20/21 and 24/25 began only in the second month after operation. (Also *see* second paragraph on p. 582.) Korschelt, however, obtained an unsegmented bud at 10/11 in 20 days and mentioned similar "Knospen" on other substrates presumably of the same series. Hescheler (1896, p. 242) found that the anterior half of an autotomized specimen of ***Allolobophora chlorotica*** (Savigny) 1826, regenerated only after nine months (autotomy in November, regeneration in August).

Liebmann (1942) apparently found no effect of season on posterior regeneration in *E. foetida* though other species failed to regenerate in winter months. In 31 October operations at 12/13–26/27 (Morgan, 1895, p. 458) no regeneration was obtained, while in 8 January operations at 19/20–27/28 one regenerate was secured. No data available as to season of Morgan's 1897 operations and Korschelt's 1898 operations. Korschelt's bud at 10/11 was obtained in August.

(i)—L20S Level of cut when last twenty segments were excised.

In the first exceptional case the small regenerate, produced in 65 days at 32/33, had the external characteristics of an inverted buccal segment, the prostomium ventral rather than dorsal. The second exceptional regenerate, produced at 34/35 in 65 days, was differentiated into six segments of which the proximal five were normally setigerous. The terminal segment had all the external characteristics of a normal buccal segment with normal prostomium.

At each level from 92/93 to 124/125 one, two, three, or ten segments were removed. Every specimen (100 +) regenerated. Regenerates produced after removal of ten segments were hypomeric, as at levels anterior to 92/93. After removal of one to three segments, regenerates were usually equimeric or hypermeric. In one specimen, number of segments in regenerate and substrate totalled 126, one more than the maximum hitherto recorded for the species.

Attempts at removal of a portion only of the so-called anal segment also resulted in hypermeric regeneration.

B. After a previous regeneration

Attempts to test for effects of previous regeneration were made in several series. In E13 and E14, the first two and three segments were removed and the substrates were allowed to regenerate for 14 days, at which time the worms were depositing faecal pellets of paper indicating that the digestive system was functional. Two days later posterior portions were removed at 8/9 or 15/16 (all anterior regenerates equimeric). All substrates of eight segments died without regeneration. Three fifteen-segment substrates survived until preservation 65–95 days later. Each had a regenerate at 15/16. One was abnormal. The second, though two mm., long, was metamerically undifferentiated and with the terminal portion sculptured into the shape of an inverted buccal segment as in the exceptional regenerate at 32/33. The other regenerate was marked off into four segments but setae were unrecognizable externally and the terminal portion was unsculptured (no indication of prostomial demarcation, buccal or anal invagination).

In E43 the anteriormost eight segments were removed. Six worms survived this operation. Forty-five days after the first operation, posterior portions were removed at 35/36. Three survivors of the second operation regenerated in the time available. No differences were noted between those regenerates and others produced at 35/36 by substrates that had not previously regenerated.

C. After starvation

Worms that had been starved seemed to regenerate as well as those which had not been starved prior to operation. As a result of several months starvation the chloragogue layer on the gut of substrates was drastically reduced. The masses of coelomic corpuscles which had been readily visible in young tail regenerates of animals operated upon shortly after collection, were not noted in those regenerating after weeks of starvation.

Discussion

A zero level posteriorly for tail regeneration apparently has been indicated by Liebmann (slightly to left of c, 1943, p. 601) and by Moment (at 90/91, by extra-

polation in curve of Fig. 2, 1946, p. 406). New results presented above show there is no zero level posteriorly.

Liebmann and Moment apparently also found that production of new segments ceased at or shortly after the first month. The author's results agree with those of Michel (1898) and some of Morgan (1895–1906), in showing that production of segments may be continued longer than a month. After reduction of the regenerative growth zone to the small anal region characteristic of the normal adult, further segment formation apparently is continued by a slow terminal method.

Establishment in tail regenerates, at the same level and in the same experimental conditions, of different types of growth zone (ventral, large or small terminal, see Gates, 1948), with different capacities for segment formation, suggests that the variation may be due to the condition of the operated animals (note also considerable variation in number of segments regenerated at each of Moment's levels, 1946, p. 496).

In favorable conditions, and at levels from 40/41 backwards, season apparently has no influence on ability to regenerate posteriorly (by anterior substrates) in *E. foetida*, although seasonal inability to regenerate posteriorly has been reported from other Lumbricid species by Liebmann (1942, for *Lumbricus terrestris*) [1] and by Abeloos and Avel (1928, for *Allolobophora longa* and *A. terrestris*). Effect of season on posterior regeneration at levels in front of 40/41, in *E. foetida* as well as other Lumbricids, remains to be determined (see note h, Table II).

The hypomery of tail regenerates at anterior levels and after removal of ten or more segments posteriorly, in the author's experiments, is in accord with D'Arcy Thompson's rule "that regeneration tends to fall somewhat short of a ***complete*** restoration of the lost part; a certain percentage only of the lost tissues is restored" (1942, p. 274). Equimery and hypermery at levels behind 92/93, when three or fewer segments were removed, provide, in earthworms, a second exception to the rule.[2] Michel (1898) had a single case of hypermery after removal of five segments (see Table II), but no information is available to indicate that the specimen was normal and complete at time of amputation. Moment (1946) obtained some hypermery in tail regenerates at 50/51 and 80/81, apparently mostly from specimens with only 80–89 segments, possibly also in some with 90–99 segments. As number of segments in juveniles 7.5 + mm. long may be 85–115 (Gates, 1948), and usually is somewhat over one hundred by six months (Liebmann, 1943, p. 600), Moment's worms with the smaller numbers of segments, 80–89 or even 90–99, may have been amputees, possibly with enteroparietal healing and no regeneration (see Gates, 1949b).

Posterior heteromorphosis, i.e., regeneration of a head at the posterior surface of an anterior substrate, hitherto has been unrecognized in the Lumbricidae. Such heteromorphosis has, however, already been recorded from species of two other families of earthworms, in the Glossoscolecid *Criodrilus lacuum* by Janda (1926) and in the Megascolecid *Perionyx excavatus* by Gates (1927). In that respect, at least, pattern of regenerative capacity now appears to be similar in the three families.

[1] There are no valid records of posterior regeneration by this species in any season!

[2] The first exception; equimery of anterior regenerates in the anteriormost portion of the axis (see part I).

Posterior heteromorphosis having been recognized in *E. foetida*, it is now possible to suggest cases in which similar results may have been obtained by previous investigators, though unrecognized. Among such cases may be mentioned: the "majority" of Liebmann's animals operated at 30/31 which died "after regenerating short pieces," and Korschelt's regenerates at 12/13 (+ ?), 9/10 (or 10/11), 8/9, 7/8 and 6/7 (see Table II). In fact, the only evidence available to indicate that posterior regenerates in *E. foetida*, at levels anterior to 20/21, are other than cephalic, is Korschelt's figure (1907, Fig. 50A, see note (a), Table II) of a specimen about which nothing else is known. In spite, then, of the failure of the present and some other authors to obtain any regeneration anterior to 20/21, there appears to be a possibility of heteromorphosis as far forwards as 6/7. This is supported, to some extent, by the heteromorphic heads regenerated by reversed grafts [3] in three different Lumbricid species: *E. foetida* (Hazen, 1899), *Allolobophora longa* (Ruttloff, 1908, and Leypoldt, 1910), and *A. caliginosa* (Crowell, 1937).

Cases of heteromorphosis from reversed grafts have all been at rather anterior levels, 7/8 (Hazen and Ruttloff), region of 8/9–7/8 (Leypoldt), 12/13 (Crowell). Levels of heteromorphosis, involving a single transection only, were somewhat more posterior. The level for Janda's case, in *C. lacuum*, was 22/23. The level for *P. excavatus* was not published, but almost certainly was in front of 20/21 and probably behind 12/13. Formation of heteromorphic structures in *E. foetida*, as far back as 34/35, accordingly had not been anticipated, though expected in a more anterior region where no regeneration (except uncounted, inhibited buds) was obtained by the author.

Regeneration of heteromorphic heads at levels 6/7, 9/10, 32/33, 34/35, etc., is believed to indicate similar regenerative ability at all of the intermediate levels.

Number of segments in heteromorphic heads on reversed grafts usually has been small for the level: 2 segments at 7/8 (Hazen), 3 in region of 8/9–7/8 (Leypoldt), 3, 4, and 5 at 7/8 (Ruttloff), unrecorded for Crowell's regenerates. However, Janda's regenerate, following simple transection only, at 22/23, had 16 segments, and Korschelt's at 9/10 had seven segments. Homomorphic head regenerates may have 16 segments in *C. lacuum* and nine segments (the maximum hitherto recorded for the Lumbricidae) in *E. foetida* (see part I). It would not be surprising if further work demonstrates an ability to produce at any particular level, in the proper conditions, as many segments in heteromorphic as in homomorphic head regenerates.

Regeneration of a heteromorphic head at 34/35 where a homomorphic tail may also be regenerated, indicates for *E. foetida* a region of bipotential regenerative capacity for posterior regeneration, such as has already been demonstrated in part I for anterior regeneration. For the present the posterior limit of the region is placed at 34/35 by the regenerate just mentioned above. The anterior limit is placed at 20/21 by the foremost recorded level of homomorphic regeneration. The region of bipotential capacity for anterior regeneration, as delimited in part I, is between 35/36

[3] At levels anterior to 20/21, regenerates from reversed grafts have usually been heads which must be considered heteromorphic with reference to original orientation of cut surfaces. The single exception was a regenerate that "erwies sich bei mikroskopischer Untersuchung als Hinterregenerat" (Ruttloff, 1908, p. 471), at 7/8 in *L. terrestris*. This case (Ruttloff's No. 60), is also of especial interest because of lack of valid records of tail regeneration by *L. terrestris* from ungrafted substrates.

and 18/19. The two regions are so nearly coextensive as to suggest possibility of identity.

Regeneration of heteromorphic heads anterior to 20/21, and regeneration of such heads behind 20/21, is not in accord with the polarization of the head aggregate and the specificity of the eleocytic aggregates postulated by Liebmann (1943).

Summary

Anterior substrates of *E. foetida*, cut exactly at intersegmental furrows, regenerated homomorphic tails at levels from 20/21 back, with no zero level posteriorly. Regenerates were hypomeric, except posteriorly and after removal of three or fewer segments, and then were equimeric or hypermeric. Heteromorphic heads, previously unrecognized in the Lumbricidae, were obtained at 34/35, 32/33, 15/16 (but only after a previous anterior regeneration). Head-like buds were formed at 16/17–19/20.

Starvation and season did not inhibit homomorphic regeneration at levels behind 40/41.

Posterior regenerative capacity in a region from 34/35 to 20/21 is characterized as bipotential, as a posterior regenerate in that region may be a homomorphic tail or a heteromorphic head.

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THE LEUCOCYTES OF ARBACIA PUNCTULATA

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Geddes (1880), whose studies supersede earlier investigations, distinguished two main types of leucocytes [1] in sea-urchins: colorless, hyaline or granular elements and colored ones with the pigment attached to cytoplasmic inclusions. He found the first to be concerned with clotting and with phagocytosis of foreign matter, observations repeatedly confirmed later; the second were surmised to serve respiration. Cuenot (1891) observed that certain granulocytes of sea-urchins liberate their albuminoid or fatty inclusions throughout the organism and claimed that these granulocytes develop from the hyaline elements (or within them), as the latter take up nutrient matter from the digestive system and transform it into the cytoplasmic granules. His later studies (1897) brought him to entirely different conclusions, viz.: that the cytoplasmic inclusions represent end products of metabolism, absorbed and eliminated by these cells, a view also held by Delage and Herouard (1913). Kindred (1924, 1926), however, supports the earlier views of Cuenot. The close connection of the granular leucocytes with the digestive tract and their penetration into its lumen through the intestinal epithelium is interpreted as a secretory function by Frenzel (1892).

Saint-Hilaire (1897) drew a sharp line between the agranular phagocytes, which he observed to ingest foreign matter and dead cells, and the granulocytes. He confirmed earlier observations regarding the abundance of the latter in and around the intestinal wall but found no indication that they are concerned with nutritive processes. A search for granulocytes in the gonads revealed no great numbers in them and no clue as to their function, leading him to the then prevailing notion that they help eliminate waste products.

The red pigment of the granulocytes, named echinochrome by MacMunn (1885), is claimed by him to be an oxygen carrier, a view shared by Griffiths (1892). Cannan (1927) found it to be an activator rather than a carrier of oxygen. Crude extracts of echinochrome are claimed by Friedheim (1932) to increase the respiratory rate in sea-urchin eggs; this, however, is denied by Tyler (1939) for the chemically pure substance. Echinochrome was also found to be present in the eggs and test of *Arbacia punctulata* (McClendon, 1912).

Comparative hematological studies on representatives of tunicates, insects, annelids and the coelenterate *Tubularia* (Liebman, 1946, 1947 *et ante*), have revealed two main types of leucocytes in these organisms: ameboid, agranular *phagocytes,* and non- or poorly ameboid, granular elements having a nutritive function

[1] This term is applied to all blood cells excluding those containing hemoglobin: the erythrocytes. "Granulocytes" designates elements with endogenous, regularly occurring cytoplasmic inclusions, irrespective of their size.

and hence termed by the writer *trephocytes*. Each kind was found to exhibit distinctive morphological and physiological characteristics, having frequently a separate locus of origin. The present investigation disclosed that conditions in Arbacia do not differ fundamentally from those found in other invertebrates studied and thus an extension of the scheme outlined above is permitted.

Material and Methods

Specimens for cytological studies were collected and preserved at the Woods Hole Marine Biological Laboratory during June and July, 1945. Additional material was secured and observations in the living state were made at the end of July, 1948. All were performed on fully grown, apparently healthy, individuals.

The leucocytes of Arbacia undergo profound changes during fixation and in some cases disintegrate beyond recognition. In addition, the pigments which characterize the various kinds of trephocytes dissolve in the fixatives and usually attach themselves to non-colored or differently colored elements, which easily leads to further confusion and misinterpretations. To avoid these pitfalls, observations on living leucocytes were made the basis of the present investigation. The blood (perivisceral fluid) was consequently studied extensively in the living condition shortly after removal, and also in hanging drop cultures kept at room temperature.

Blood-smears prepared in the conventional way proved of little value. The only method giving satisfactory results was a modification of the formalin-vapor technique described earlier (Liebman, 1945a) : the blood is spread in a thick layer on a slide and placed in a moist chamber, a Petri-dish lined with wet filter-paper serving well the purpose. After 30 minutes the cover of the dish is slightly lifted and a wad of cotton soaked with formalin placed near, but not in contact with, the slide. Fifteen or twenty minutes later the slide is removed, air-dried and stained. The leucocytes, which become active in the moist chamber, are fixed by this method in the characteristic forms as they appear in life. Hanging drop cultures were also successfully fixed by this method. Preparations from blood, quickly withdrawn from the body and injected directly into the fixative, proved useful for some purposes since this method fixes certain elements in the same condition as they are found within the organism; it is referred to in the text as "whole-blood" method.

Preparations showing leucocytes fixed *in situ* are preferable where nuclear structures have to be considered. Aside from pigment displacement, mentioned earlier, the leucocytes—especially the granular kind—usually appear here in a contracted, more or less spherical shape, which they seldom assume in life; this, however, proved of considerable advantage when measurements had to be made and these were performed on said material. In fixing tissues or whole organisms, Zenker-formol produced the best results and was used extensively, except when other considerations required different methods.

Wright's blood-stain, strongly diluted in distilled water, and Mallory's connective tissue stain gave the most satisfactory results. Mallory's stain produced particularly sharp pictures of the minute nuclear and cytoplasmic structures, as well as a differential staining of the trephocytes after Zenker-formol fixation.

I am greatly indebted to Dr. Ross F. Nigrelli for his generous help and advice in preparing the photomicrographs.

Observations

A. The trephocytes

Calculations based on a count of 1608 cells from the perivisceral fluid of four individuals showed 50.4 per cent to be trephocytes, the remainder phagocytes. However, the number of trephocytes in the whole organism was probably higher, as they were practically the only leucocytes encountered in the ovaries; reports also agree that they are predominant in the digestive tract.

Observations *in vitro* revealed three kinds of trephocytes, having respectively green, red or colorless cytoplasmic inclusions. For sake of brevity they are referred to here as green, red or colorless trephocytes.

The green trephocytes. These are the least numerous, constituting 10.2 per cent of all trephocytes. They are the smallest in size, with an average diameter of 7 μ and a range of 6–8 μ. The nucleus is small, round and lacks a nucleolus but,

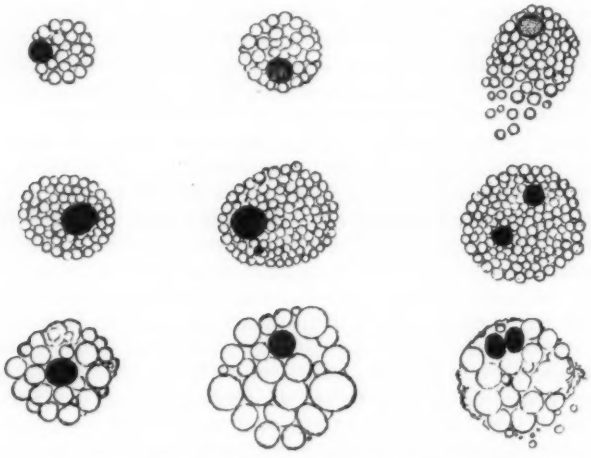

Figure 1. Trephocytes from an animal fixed in toto; upper row: green variant; median row: red variety; lower row: colorless variety. Camera lucida drawing; 1650 ×. In each row the element at left represents a young form, that at right an old one.

in contrast to the two other kinds, it shows minute chromatin granules (Fig. 1, first row). The cytoplasmic inclusions are fine, rather uniform in size and of a lemon-green color in the living cell. They are moderately eosinophilic or, in a small proportion of cases, chromophobic. With Mallory the granules stain reddish-orange, as does the nucleus.

In the living state, the green trephocytes occasionally contain a number of red cytoplasmic granules like those found in the next variant.

The red trephocytes (Fig. 1, second row) were the most numerous, comprising 48 per cent of the group's total. They average 9.2 μ in diameter with a range of 6.7–12 μ. Their anucleolar nucleus is somewhat larger than that of the green kind, occasionally dumb-bell shaped or fragmented into two portions. When stained, it

assumes a flat, structureless aspect. The cytoplasmic granules are uniform in appearance and of the same size as those previously mentioned, but often coarser in the largest elements. In life they are of a brownish-red color due to the presence of echinochrome. They stain light-green with Wright and a brownish-yellow or light blue with Mallory.

The colorless trephocytes comprised 41.8 per cent of the total during the period of investigation. They are the largest kind (Fig. 1, third row), averaging 10.2 μ in diameter with extremes ranging from 6.0–13.4 μ. The nucleus is minute, often smaller than that in the green kind, anucleolar and devoid of structures; in the larger cells it is frequently pycnotic, fragmented or occasionally missing, having apparently dissolved in the cytoplasm. The cytoplasmic inclusions are colorless or faintly greenish in life, irregular in size but predominantly very coarse. They are very strongly basophilic, occasionally slightly metachromatic; Wright's stain makes them appear almost black and completely opaque. With Mallory they stain a light-blue.

Chemical nature of the inclusions. The difficulties encountered in the cytological study of the trephocytes apply also to the cytochemical approach, hence our examinations should be considered of a preliminary character and the information gained rather of a tentative nature. Definite conclusions could only be drawn after a special study of the problem, with new or specifically adapted methods.

Routine techniques did not reveal the presence of osmio- or sudanophilic matter in any of the trephocytes. Orange-yellow coloration after Mallory's staining, assumed by some authors to suggest phospholipins, appeared in the green, red and occasionally also in the colorless inclusions. Glycogen, as indicated by Langhans' iodine-method and Best's staining, and checked with the sputum test, was only very rarely found. The strong basophilia of the colorless inclusions suggests the presence of ribonucleic acid and their positive reaction towards mucicarmin—a mucin. The color of the inclusions of the red trephocytes is generally attributed to echinochrome.

Common characteristics of the trephocytes. The regular presence of endogenous granules is *per se* a common feature of the trephocytes. Another is the characteristic nucleus.

Additional features peculiar to all trephocytes are their function, to be discussed later, and the lack of phagocytic capacity.. Observations *in vitro,* both on freshly drawn blood as well as on cultures, in no case revealed ingestion of foreign matter, although in the older cultures extensive phagocytosis was evident in other cells. Similarly, in the fixed material no indication of phagocytosis on the part of the trephocytes was found.

Another common peculiarity of all trephocytes is their way of locomotion. They move by emitting blunt lobopodes into which the granules follow quickly. When moving, the cells usually assume an oblong appearance, frequently with a trail of granules or cytoplasm in their wake (Fig. 2), which gives them a striking resemblance to rat-tailed maggots.

As against these common features, the composition of the granules thus appears to be the main factor distinguishing the three kinds of trephocytes. Even here, however, the limits do not appear to be fast and absolute; this is suggested by some tinctorial as well as cytochemical characteristics common to all granules.

Genetic relationship of the trephocytes. The relatively well differentiated nucleus, the small size and the rather low frequency of senile, disintegrating forms, suggest that the green trephocytes are the youngest of the three variants. Of the green trephocytes, the chromaphobe forms are the smallest, have the largest and best

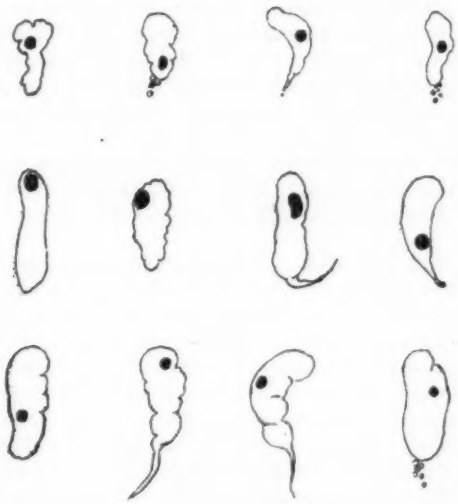

FIGURE 2. Outlines of living trephocytes in motion; from a hanging drop culture; camera lucida; 1000 ×. Upper series: green; middle series: red; lower series: colorless trephocytes.

differentiated nuclei and are consequently regarded as the youngest in this group. Also, chromophobia of the inclusions is peculiar to very young cells and has repeatedly been observed by the writer in the earliest developmental stages of granulocytes (trephocytes of annelids, eosinophils of fishes and amphibians). The largest forms amongst the green trephocytes, on the other hand, generally represent the

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PLATE I

All figures are unretouched photomicrographs of sections of ovaries fixed in Zenker-formol and stained with Mallory's connective tissue mixture.

FIGURE 1. Cross-section from an ovary, showing the interovular spaces filled with disintegrated trephocytes. The clear areas around the oöcytes are due to shrinkage; 600 ×.

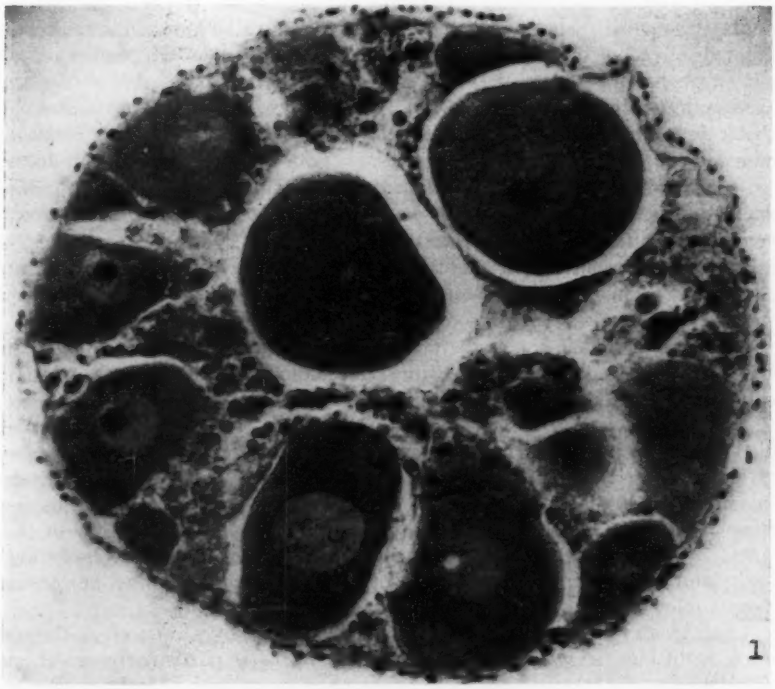
FIGURE 2. A compact mass of partly disintegrated red trephocytes surrounded by oöcytes; the dark spots in the mass represent nuclei; 450 ×.

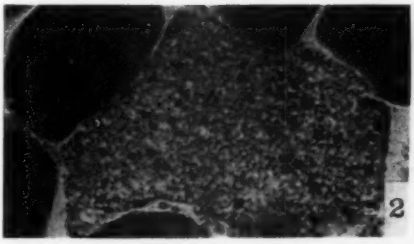
FIGURE 3. An oöcyte showing uptake of trephocytic granules along its free (upper) side; 650 ×; note lack of cell-wall and continuous stream of granules at the ingesting site. In this, as in the following two figures, the lower edge of the picture corresponds to the site of attachment of the oöcytes to the ovarian wall.

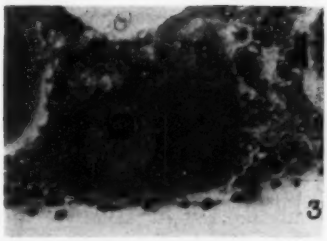
FIGURE 4. A young oöcyte in the process of uptake of larger droplets and a nucleus (along the upper right side); 800 ×.

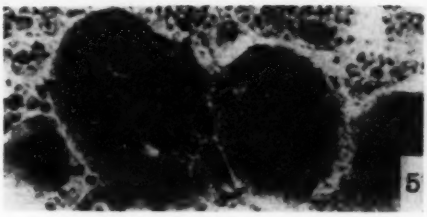
FIGURE 5. Two oöcytes engorged with trephocytic granules; 800 ×. A close inspection shows small perforations in the ovular walls.

PLATE I









oldest elements, showing most frequently pycnotic nuclei and signs of cellular disintegration. This scheme, if not too rigidly applied, holds also for the two other variants, the smallest usually representing the youngest, the largest the most mature individuals of each group. Among the three groups, the colorless trephocytes show the most pronounced signs of maturity and disintegration.

Elements which show green and red inclusions are not infrequent, indicating the probable origin of the red variant from the green one. The colorless forms often exhibit a more or less green hue, suggesting also a possible development from the green kind. Tinctorially, on the other hand, the colorless and red granules occasionally show common characteristics. Despite extensive search, however, we have not found in the perivisceral fluid colorless elements with clear-cut transitional or mixed characteristics, as is the case with the trephocytes containing green and red granules. This does not preclude the possibility that the transformation into colorless elements takes place in some organ.

Neither in the blood nor in the tissues studied (gonads and adjoining tissues) were there any indications suggesting development of the trephocytes from phagocytes, nor was there any indication of a common origin.

The function of the trephocytes. Distintegration of all three variants, accompanied by a scattering of the cellular components was observed both *in vitro* as well as in fixed material, in the blood as well as in tissues. This is usually preceded or paralleled by loss of staining capacity of the nucleus or, more rarely, by its becoming pycnotic. Occasionally the cell components simply fuse into one or several droplets; this was observed mostly in the colorless trephocytes. Considering the ubiquity and the high frequency of the processes described, they must be considered as normal phenomena.

The ultimate fate of the trephocytes and their derivatives, as well as the nature of their inclusions, suggest a trophic function. This is particularly evident from their relation to the growing eggs and to the phagocytes, both objects of close study in the present investigation.

The ovaries were found to be densely packed with trephocytes, most of them in a partly or fully disintegrated state. This cellular brei surrounds the oöcytes from all sides except where they border the ovarial wall, as is the case in the youngest elements (Plate I, Fig. 1). It appears to be well attached to the ovules, as witnessed by the difficulty of separating it from the oöcytes in isolates. In some places the trephocyte masses form extensive, uninterrupted islands in the ovary (Plate I, Fig. 2).

Only the red and colorless variants of trephocytes were found in the ovaries. Because of their disintegrated state, it was impossible to submit them to a differential count, the general impression being, however, that their proportion roughly corresponds to that in the blood.

Suitable preparations reveal that the young oöcytes take up granules and droplets of the cellular brei surrounding them. This apparently takes place to a great extent by a process of fusion: the ovular membrane in the areas concerned is very thin, discontinuous or missing and the granules form an uninterrupted stream from the exterior practically up to the nucleus (Plate I, Fig. 3). Occasionally large droplets, some still showing unmistakable marks of nuclei, are found to be taken up in this manner by the oöcytes (Plate I, Fig. 4). On the other hand, very small

granules appear to penetrate through the ovular membranes and are frequently found sticking in them (Plate I, Fig. 5). Extremely small perforations are also detectable in the wall of the young oöcytes, as shown in the same figure, but these are not necessarily preformed openings but may represent empty spaces from which granules have been dissolved in the process of fixation, dehydration, etc. The result of all these activities is that the young oöcytes frequently appear gorged with granules (Plate I, Fig. 5), whose physical and tinctorial appearance leaves little doubt regarding their origin from the trephocytes.

The phenomena described indicate that a considerable proportion of the egg's substance consists of material taken up in corpuscular form and derived from the trephocytes. Since a large fraction of this material can be traced to their red variety, the possibility should not be precluded that the echinochrome found in the eggs of Arbacia also has its origin in the trephocytes.

B. The phagocytes

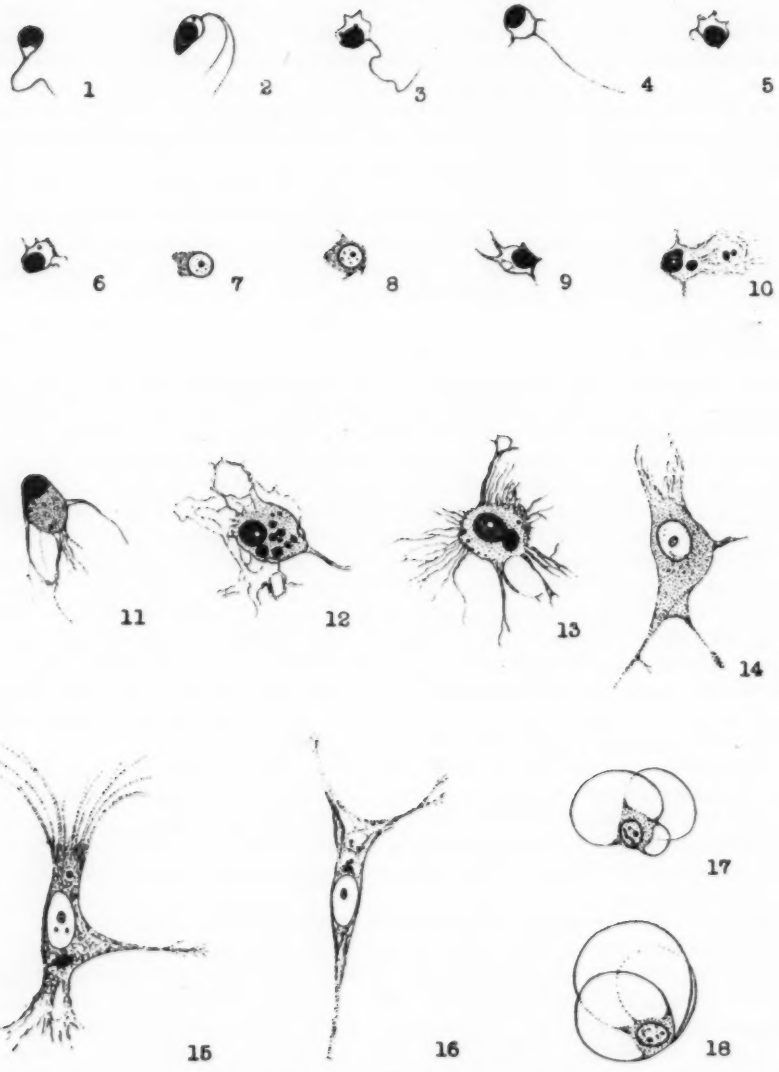
The variety of these elements in the species studied, as well as in the echinoderms in general, has hardly its equal in the animal kingdom. Still, even more clearly than in the case of trephocytes, the present studies reveal that they are merely developmental and functional stages of the same cell, some possibly representing reversible adaptations to the medium in which they are found. They are here classified into four groups, according to their outstanding characteristics: (1) Flagellated forms, (2) Ameboid forms, (3) Fibroblasts and (4) Petaloid forms.

The flagellated phagocytes represent the smallest and apparently youngest stages of this group (Table II, Fig. 1–4). In the living state they appear as spherical elements gyrating and moving briskly among the other blood cells. When fixed, they show one—rarely two— flagella and a spherical, ovoid or piriform cell-body. The nucleus is relatively large and nucleolated, the chromatin fine. (In smears, the nucleolus of these, as well as of other small elements, is rarely visible.) Both *in vitro* as well as in fixed preparations the cytoplasm occasionally shows trephocytic granules, suggesting a certain phagocytic capacity.

Flagellated elements are frequently observed *in vitro* to settle, lose their flagellum and become ameboid. Figures 3, 4 and 5–8 (Plate II) represent such cells prior to and soon after this transition. Except for the absence of the flagellum and the regular presence of small pseudopods, the elements, soon after settling, hardly differ from the flagellated forms. The small size, high nuclear-cytoplasmic ratio, and poor phagocytic capacity, as well as some other characteristics, suggest a similarity of the flagellated and young ameboid forms to the vertebrate lymphocytes.

The ameboid phagocytes. Between the smallest, aflagellar, ameboid forms and the largest elements—the macrophages—innumerable transitions can be observed both *in vitro* and in the fixed preparations (Plate II, Figs. 5–13). They are all characterized by a relatively large nucleus of a round, spheroid, occasionally bean-shaped form, and fine chromatin. A large nucleolus, sometimes two or three, is always present. The hyaline cytoplasm forms long slender pseudopods continuously changing shape and configuration. This ameboid capacity seems to increase with the size of the cells, the latter again being, in general, an expression of their physiological age. Small and medium-sized forms were most commonly encountered in the blood during the present investigation. The whole group cor-

PLATE II



Various forms of phagocytes; camera lucida drawings; 1300 ×. Figures 1–6 and 9–16 from a formalin-vapor preparation. Figures 7, 8 from an animal fixed *in toto;* Figures 17, 18 from a whole-blood preparation. The black matter in the cytoplasm represents ingested trephocyte material.

responds to the vertebrate mononuclears; the largest forms show in most respects a similarity to the macrophages of vertebrates and other invertebrates.

The fibroblasts. In hanging drop cultures, elements closely resembling vertebrate fibroblasts (Plate II, Figs. 15, 16) appeared within 24 hours. Transitional forms between them and the macrophages were observed both *in vitro* and in fixed material (Plate II, Fig. 14) and it is highly probable that they develop from macrophages. Without implying that they correspond in all details to their vertebrate counterparts, their general appearance unmistakably justifies their being classified as fibroblasts. The nucleus, except for being elongated, is similar to that of the macrophages; the cytoplasm is fibrillar or reticular, a characteristic appearing also in the macrophages in regions where long pseudopods are formed.

The petaloid phagocytes. Another variety of phagocytes is illustrated in Figures 17, 18 (Plate II). Their ectoplasm forms several roughly semicircular membranes or flaps which continuously change shape and position. The nucleus of this form does not differ from that of the ameboid phagocytes. Similar phagocytes have been described in earthworms where their membranes are oblong and arranged around the cell somewhat like petals.

Observations on freshly-drawn blood as well as on whole-blood preparations indicate that this form is very common in the perivisceral fluid. *In vitro* it was only found in a suspended condition and observations lead to the conclusion that it should be considered an adaptation to a floating way of existence, the membranes—apart from other functions—acting to increase the buoyancy, as in the case of planktonts. When the cell settles on a substrate it changes into an ameboid form, the membranes often persisting for a considerable time. A similar change was observed in the phagocytes of earthworms, when they come in contact with a solid surface (Liebman, unpublished observations) and in human leucocytes (Bessis and Bricka, 1949). There are indications that the ameboid form of Arbacia, if detached from the substrate, turns into the floating variety.

Common characteristics of the phagocytes. The nucleus is single, relatively large and never missing; its form more or less spherical, ellipsoid or bean-shaped. A large nucleolus, occasionally accompanied by one or two smaller ones, is always present.

The cytoplasm is hyaline and devoid of endogeneous inclusions. Ameboid movement appears in all forms in the presence of a substrate and increases markedly with the volume of the cytoplasm. Phagocytosis is present in all stages.

Function of the phagocytes. Though the phagocytes are known to be involved in several other functions, the present study is solely concerned with their capacity to take up formed matter. Under natural conditions this, except for occasional bacteria, consists almost exclusively of trephocytic granules or whole cells.

The flagellated forms were occasionally found to contain trephocyte granules of the green, red, or colorless variety in their cytoplasm. Because of their rapid movements, it could not be ascertained whether these are taken up by pseudopods or with the aid of the flagellum.

The ameboid forms phagocytose in the familiar way of engulfment with the aid of pseudopods. Generally, their phagocytic capacity appears to increase with the size of the cell or, more correctly, with the amount of the cytoplasm. In the fibroblasts, on the other hand, despite their relatively large size, the phagocytic capacity appears to be considerably reduced.

The petaloid forms show the highest phagocytic activity. This, however, is not necessarily due to an inherent quality but is probably owing to the ability to operate unimpeded in all three dimensions, in contrast to corresponding ameboid variants, phagocytosing on a substrate.

Whole, living trephocytes were repeatedly observed *in vitro* to be attacked by single, occasionally several, petaloid phagocytes. The trephocytes are caught by the flaps moving their surfaces close to each other and finally over the prey, somewhat in the manner in which a ball is caught in two hands. The engulfed trephocyte is seen moving vigorously within the phagocyte for a considerable time but ultimately undergoes gradual digestion, though its movements occasionally help it to "escape." This peculiar form of phagocytosis is also common within the organism, and whole-blood and *toto* preparations show a relatively high percentage of phagocytes containing intact trephocytes or some in various degrees of digestion (Fig. 3).

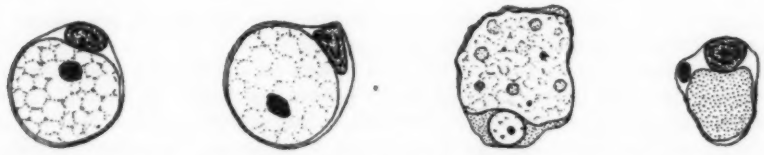


FIGURE 3. Phagocytes with engulfed intact and digested trephocytes; from whole-blood preparations; camera lucida; 1650 ×.

The uptake of trephocytic matter and the engulfment and digestion of living elements cannot be considered as phagocytosis in the accepted sense, which usually implies ingestion of noxious substances to be eliminated from the organism. It represents a normal way of nutrition for the phagocytes, and the phagocytosis of live trephocytes—cannibalism.[2] This, of course, should not imply that the phagocytes do not ingest matter destined for elimination, like dead cells, bacteria, or introduced foreign substances.

DISCUSSION

Previous studies on trephocytes (Liebman, 1946, 1947) have revealed that formed matter liberated by them is taken up by a variety of cells: oöcytes, phagocytes and epithelial cells. This led to the conclusion that, in addition to chemical substances, the animal cell depends also on biological elements for nutrition.

The present, as well as some earlier observations, impel us to extend this notion further, by putting forward the idea that cannibalism apparently represents a normal process of cellular nutrition.

Uptake and digestion of whole trephocytes by the growing ovarian eggs are apparently common among invertebrates. In some cases (*Tubularia*) the trephocytes

[2] In the few instances where this term has been applied to cells, it is used both in a strict sense to denote ingestion of living elements by their own kind, as well as in cases where the phagocytosed cells are merely of the same orgasism. We propose to apply this term in the latter sense to all cases where a cell ingests and digests any other living cell of the same organism.

taken up by the egg are dead, thus excluding cellular cannibalism as understood by cytologists. In several tunicates investigated, however, as well as in some annelids (Liebman, *ibid.*), the trephocytes within the egg appear to be alive. In the tunicates, they seem to persist in this state for a considerable time—as within the phagocytes of Arbacia—before being digested.

In the vertebrates, lymphocytes have been reported from epithelial cells of the intestinal and renal tract of fishes and Amphibia (Loreti, 1934, 1935), the oöcytes and epidermal cells of amphibians (Liebman, 1945b, 1947), and from the epithelial cells of the digestive and respiratory tract of mammals (Andrew and assoc., 1945, 1946, 1947). Not in all these cases are the lymphocytes alive when entering other elements nor are they all digested by the host cells, but there are clear indications that cannibalism in some of these instances is not infrequent.

It is possible that Cuenot (1891) saw elements corresponding to trephocytes within hyaline cells (phagocytes) but, failing to observe digestion, assumed that the former develop within the latter.

The presence of living cells within others has been definitely established in tissue cultures. Lewis (1925) saw frog-mononuclears and epitheloid cells from tubercular rabbits engulf their own kind, but stresses the great rarity of such cases and their probable pathologic nature. Fischer and Dolschansky (1929) repeatedly observed small cells wandering in and out of stroma cells of spleen cultures. They have not observed digestion of these elements, which they consider as derivatives of the stroma cells. Some of their photographs (e.g., Figs. 5, 6) indicate however, that these wandering cells are lymphocytes and their often fragmented and pycnotic condition does not exclude the possibility that they are occasionally digested by their hosts.

The inclusions of the trephocytes are frequently colored, occasionally also the cytoplasm. Except for echinochrome, however, our knowledge of their colorants is extremely limited and even the precise role of the former in respiration, is not fully clarified.

Observations on the invertebrate erythrocytes strongly suggest that they represent a special kind of colored trephocytes. It was pointed out earlier (Liebman, 1946) that the erythrocytes of the polychaete *Cirratulus grandis* contain numerous lipid inclusions in the cytoplasm and a typical trephocytic nucleus while their fixed shape, cytoplasmic structure and presence of hemoglobin are characteristic of erythrocytes. Conditions in other polychaetes (Romieu, 1923), as well as in invertebrates generally (Ohuye, 1937), reveal similar traits in their erythrocytes. This similarity extends to the vertebrates where the first embryonic (primitive) erythrocytes contain nutritive inclusions in the cytoplasm (Maximow, 1927), and often exhibit a striking resemblance to trephocytes (Cameron, 1941, and others). Loss of the nucleus, characteristic of the mammalian erythrocytes, occasionally takes place in invertebrate red corpuscles and is a recurrent feature of the trephocytes proper. It is significant that this trait is common only to the erythrocytes and trephocytes among the blood cells.

The presence of a flagellum in the youngest stages of the phagocytes is probably due to their origin from the coelomic epithelium which is usually ciliated in the echinoderms.

Petaloid phagocytes are quite common among invertebrates. Goodrich (1920)

claims that the fine pseudopodes of the ameboid forms are either lateral views of the petals or artifacts, insisting that the only true form is the petaloid (his "membraneous") pseudopod. He also claims that the "petals" are merely folds of a continuous membrane. His extreme views are not borne out by our observations on Arbacia and earthworms.

Cuenot (1891) surmises that the movements of the flagellated forms prevent clotting of the blood. Coagulation of blood and the occlusion of wounds are generally agreed to be effected by the ameboid phagocytes forming a dense network with their fused pseudopods (pseudo-agglutination). Phagocytosis of introduced bacteria has been observed by Cernovodeanu and Henri (1906), and that of non-deposited sex products by Caullery and Siedlecki (1903).

Summary

This investigation revealed the presence of two different types of leucocytes in Arbacia: trephocytes and phagocytes. An outline of their morphology, development and physiology is presented. No genetic relationship between the two kinds could be established, a circumstance which further tends to prove the distinctive nature of each type.

Particulate matter derived from the trephocytes and essentially of a nutritive nature, is taken up and incorporated by the growing oöcytes. A considerable fraction of this material has its origin in the red trephocytes which contain echinochrome. It is suggested that the echinochrome found in the eggs of the species investigated may also be derived from the trephocytes.

The occurrence of respiratory pigments among invertebrate trephocytes is discussed. It is pointed out that the erythrocytes of invertebrates, as well as the early stages of those of vertebrates, exhibit characteristics of trephocytes. The idea is advanced that the erythrocytes represent a special kind of trephocytes.

Formed trephocytic material was seen to be engulfed and digested by the phagocytes of Arbacia. This, as in the case of the oöcytes, is regarded as a normal process of cellular nutrition and lends further support to the thesis, expressed earlier by the writer, that the animal cell takes up and assimilates particulate cellular material in addition to substances in solution.

The engulfment and digestion of living trephocytes is described and discussed and additional cases of cellular cannibalism among invertebrates and vertebrates brought forward. The conclusion is reached that cannibalism is a normal and apparently fairly common form of nutrition of the animal cell.

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CONDITIONING V. MERCENARIA FOR SPAWNING IN WINTER AND BREEDING ITS LARVAE IN THE LABORATORY

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Rapid progress in studies of the morphology, physiology and ecological requirements of lamellibranch larvae, as well as other forms, can be achieved only if these organisms are easily available for study. This is seldom so in the case of lamellibranchs because the majority have a breeding period of comparatively brief duration lasting only a few months (Lebour, 1938; Thorson, 1946). During this period the larvae can be collected in plankton tows but, unfortunately, the early stages of many lamellibranchs are so alike in size and appearance that it is often impossible to distinguish with any degree of certainty the larvae of different species, or even genera. Thus, unless these larvae can be grown to metamorphosis their specific identity may remain in doubt. It is preferable, therefore, to work with larvae raised in the laboratory from fertilized eggs because in that case their identity cannot be questioned.

In obtaining material for morphological and physiological studies of larvae the advantages of the direct method, i.e., the method of raising larvae in the laboratory, over the indirect method of collecting them in plankton and often only guessing their identity, are clear. However, because lamellibranch larvae are small, and because their free-swimming period is relatively long, they are difficult to culture. As a result, with the exception of a few successful attempts, mostly confined to commercially important species, such as oysters and mussels, few lamellibranchs have been grown in laboratories past the early veliger stage (Thorson, 1946). Therefore, the need for a simple but reliable method for culturing them is, of course, obvious. It is hoped that the simple but efficient method described in this article for conditioning the hard shell clam, *V. mercenaria*, to spawn out of season and for culturing its larvae will be applicable to many other species of lamellibranchs permitting their successful cultivation in the laboratory, where their morphological features and various aspects of behavior can be studied under controlled conditions.

Realizing the importance of having a good supply of larvae for studies of the life history of *Venus mercenaria*, Belding (1912) tried to raise them in the laboratory. He was not successful because most of the larvae in his cultures died either before they reached the straight hinge veliger stage or soon afterwards. Belding concluded that there was no practical method for raising hard shell clams to the setting stage because of the small size and delicate nature of the eggs. Several years later, nevertheless, Wells (1927) succeeded in growing clam larvae to the setting stage. Wells, however, was mostly interested in oysters and did not continue the clam work.

Obtaining clam spawn in the summer time is relatively a simple procedure. During the summers of 1933, 1934 and 1935 many clams were induced to spawn sim-

ply by raising the water temperature a few degrees (Loosanoff, 1937). However, because the spawning season of *V. mercenaria* in our waters is confined to a period of approximately 2½ or 3 months, experimenting with their eggs and larvae was necessarily also confined to the same period of time. Fortunately, since a method has been recently devised to induce oysters to develop spawn in the winter time (Loosanoff, 1945), it was decided to apply this method to clams also, hoping in this way to extend considerably the period during which ripe eggs and sperm could become available for laboratory work. The method was successful, and it is now possible to make clams spawn, releasing normal gametes, throughout the winter and spring.

The method for conditioning clams for spawning in the winter time is as follows: Clams brought from their natural beds in Long Island Sound, where the temperature of the water in the winter time is near 0.0° C., are placed in trays of running sea water having a temperature of approximately 5.0 to 7.0° C. Then, at intervals of 3 to 5 days the temperature of the water is increased by several degrees. Eventually the temperature is raised to about 20.0–22.0° C. and the clams soon become ready for spawning. The entire conditioning period usually takes about 3 weeks, but can be made even shorter, if the intervals between the increases in temperature are shortened to about one day. The clams may also be placed directly into water of a temperature of about 20.0° C., but under such a condition some mortality may occur.

Conditioned clams were induced to spawn by raising the temperature several degrees above the conditioning level. However, if the temperature was raised above 34.0° C., most of the clams usually withdrew the siphons and closed the shells. It was often noticed that spawning began during a decrease in temperature, i.e., if the temperature was first raised to about 35.0° C. and then gradually decreased.

In several instances cases of spontaneous and apparently unprovoked spawning were observed at temperatures several degrees lower than 23.0° C., which had been considered the minimum at which clams could spawn (Belding, 1912; Nelson, 1928; Loosanoff, 1937). For example, on February 7 and March 4, 1949, clams were seen spawning at 22.0° C., and on March 28 and April 5 large groups spawned in the trays having a temperature of only 21.0 and 20.6° C. respectively. In all cases both males and females were spawning, many of them quite profusely. The eggs from these spawnings were collected and cultured, the larvae reaching the setting stage.

An individual clam does not discharge all its eggs or sperm at one spawning but continues to spawn, at intervals of a few days, over an extended period. For example, in one of the groups a marked female was induced to spawn on six different occasions between February 2 and March 3. Many other clams of the same group also spawned several times. In general, this group provided spawn for a period of approximately 5 or 6 weeks, before the majority of the clams became spent.

Not all the eggs discharged by the spawning females possessed the same vitality. Probably some clams were compelled by the strong temperature stimulation to abort the eggs even if the eggs were not fully ripe. Such eggs usually developed into feeble larvae which soon died. The last batches of eggs discharged by virtually spent females also gave feeble larvae that grew slowly and showed a high mortality. With a little experience, however, an investigator can learn to recognize various types of spawnings and select only those batches of eggs that are suitable for cultivation.

The eggs to be used for cultivation of the larvae were fertilized as soon as they were discharged. To separate them from the debris accumulating in the spawning dishes the water containing the eggs was run through a Number 80 stainless steel testing sieve, having openings of 177 microns. These openings were large enough to let the eggs surrounded by membranes pass through but retained the larger particles. After that the egg suspension was filtered once more through another sieve, which was fine enough to retain the eggs but let the water containing the sperm, blood cells, etc., pass through. The retained eggs, now free of all impurities, were placed in fresh sea water in glass hatching jars of about 14 liters' capacity, which were continuously aerated.

The eggs and later young larvae remained unattended until they developed into early veliger. Then the water in the jars was renewed every second day. To accomplish this the content of the jars was strained through fine sieves, which retained the larvae but let the water pass through. The jars were then filled with new water and the larvae returned to them.

To feed the larvae, small quantities of mixed plankton cultures, consisting primarily of forms of less than 5 microns in size, were added daily to each jar. The food cultures were grown during the winter in large aquaria, and in the summer in the outdoor, concrete tanks filled with sea water and enriched with a commercial fertilizer (Loosanoff and Engle, 1942). Within a few days extremely rich, mixed cultures consisting chiefly of phytoplankton were available for feeding the larvae of the clams. This food was also used to feed numerous cultures of larvae of two other species of clams, *Mya arenaria* and *Mactra solidissima*, and three species of oysters, *Ostrea virginica*, *O. gigas*, and *O. lurida*, which were grown to metamorphosis at our laboratory.

When the larvae were reaching the setting stage old oyster shells were placed on the bottom of the jars to provide a place for attachment, or the larvae were transferred to special aquaria on the bottom of which a layer of sand was spread.

A description of the development of the clam egg and of the morphology of larvae has already been given by Belding (1912), on the basis of material which he found in plankton. Therefore, it is not necessary here to go into most of the details. Instead we shall offer a brief account of the clam development from fertilized eggs to the dissoconch stage, as observed under laboratory conditions at a temperature of about 22.0° C.

The egg of the clam measures from about 70 to 73 microns in diameter (Fig. 1, A). It differs from the eggs of some other lamellibranchs in that it is surrounded by a thick gelatinous membrane the diameter of which usually is within the range from 163 to 170 μ. It was noticed on many occasions that this membrane continues to surround the egg past the blastula stage and, sometimes, until trochophore larvae are formed. If at fertilization spermatozoa are numerous, many can be seen imbedded in the outer portion of this membrane (Fig. 1, A).

The fertilized egg will reach the two-celled stage in about 45 minutes (Fig. 1, B), and the four-celled stage is reached in about an hour and a half (Fig. 1, C). In about 6 hours the embryo becomes a well-developed, rotating, ciliated blastula. The early gastrula stage is reached after 9 hours, and finally the larva enters into the trochophore stage which is reached about 12 hours after fertilization (Fig. 1, D). This form, roughly pear-shaped, moves through the water with a spiral motion,

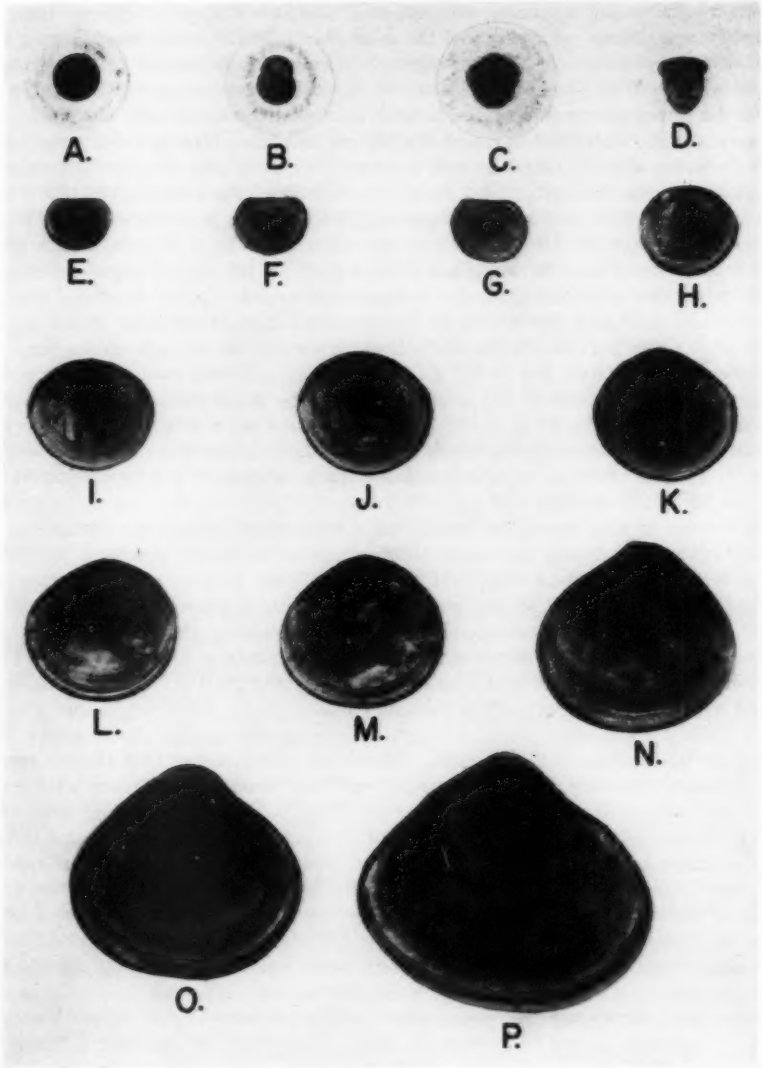

FIGURE 1. Different stages of development of clams through larval and early post-larval stages. × 112. Description in text.

propelling itself by a circlet of cilia around the anterior end, aided by a velar tuft of longer cilia at the extreme anterior end. In this respect it differs from the gastrula stage during which almost the entire body of the larva was covered with small cilia. Furthermore, the trochophore larva begins to form a primitive mouth and develops a shell gland. The larva at this time measures about $90 \times 65\ \mu$.

As the development progresses, a small thin shell is secreted by the shell gland and is gradually extended to cover the entire animal. This usually occurs from 24 to 36 hours after fertilization and the larva is now in the early veliger or early straight hinge stage (Fig. 1, E). Its size at this time is approximately $105 \times 80\ \mu$. About 8 to 12 hours later a true veliger or straight hinge stage is attained (Fig. 1, F). At this stage the larva, which is approximately $110 \times 90\ \mu$ in size, becomes quite a proficient swimmer using for this purpose its highly developed velum.

If conditions are favorable, the veliger continues to grow reaching the size of about $122 \times 98\ \mu$ by the end of the fourth day, but still remaining in the straight hinge stage (Fig. 1, G). By the sixth day it is already in the early umbo stage and measures approximately $154 \times 143\ \mu$ (Fig. 1, H). In 8 days some rapidly growing larvae may reach the size of $205\ \mu$, while the average are in the medium umbo stage measuring $195 \times 178\ \mu$ (Fig. 1, I). About 10 days after fertilization many individuals in good cultures are in the late umbo stage, measuring about $214 \times 192\ \mu$ (Fig. 1, J), and after 12 days some of the mature, ready-to-set larvae may be as large as $227 \times 210\ \mu$ (Fig. 1, K).

Just prior to this stage clam larvae begin to undergo prominent changes. The velum begins to disappear and some of its functions are taken over by a foot which is covered with numerous cilia. At first this ciliated foot aids in swimming, but gradually is used more in gliding over the bottom and in crawling. Eventually the velum entirely disappears, thus ending the free-swimming period.

In most cultures many larvae reached the setting stage in about 12 days. However, in some cultures grown at a temperature of about 24.0° C. metamorphosis occurred in ten days. The smallest metamorphosed clams were only about 210 microns. They were often seen attached by the byssus to the shells which were placed on the bottom of the aquaria. Thorson (1946) states that the veligers of *Venus gallina* also very often set when they are only about $210\ \mu$ although the length of the prodissoconch varies between 210 and $225\ \mu$. We found even greater variations in the size of the prodissoconch shell of *Venus mercenaria* because some of them were as large as $240\ \mu$. Nevertheless, none of the prodissoconch of our cultures ever approached the size of $320\ \mu$ as reported by Sullivan (1948) for the Venus larvae of Malpeque Bay. However, as Jørgensen (1946) showed, the size of the larvae at the time of setting may vary considerably according to the conditions of the environment and, therefore, the measurements made at setting are only of relative importance.

After metamorphosis the young clam begins to form the adult or dissoconch shell. Successive stages of growth showing the increase in size and formation of adult shell are given in Figure 1, L, M, N, O and P. The sizes of these small clams were 240×223, 260×245, 313×308, 366×340 and $463 \times 423\ \mu$ respectively. The oldest individual shown was 28 days counting from the day of fertilization.

Significant variations in the sizes of the individual larvae of the same cultures were very often noticed. For example, while some of the largest larvae were ap-

proximately 210 μ in size and were ready to metamorphose, the other larvae of the same culture were only 150 or 160 μ long and were still far from the end of the free-swimming stage. Sometimes, because of overcrowding, a difference in temperature or other factors, the average size of the larvae of two parallel cultures carried in separate jars would also show significant differences.

Clam larvae were not too selective in their food, surviving and growing rapidly on many micro-organisms instead of being confined to a few forms. The exception was when the clam larvae were fed almost a pure culture of a certain species of Chlorella. The larvae so fed grew more slowly and showed a heavier mortality than those which were fed mixed plankton cultures.

We found that the method for staining oyster larvae, which we described some time ago (Loosanoff and Davis, 1947) is also applicable to clam larvae. By using a weak solution of Neutral Red, clam larvae were stained and thus became easily distinguishable from the normal individuals. It is believed that this method will help, later on, to study the dispersal of larvae from the place of origin, their rate of growth under natural conditions, etc.

In conclusion it may be said that by following the few simple principles and rules given in this article mature sperm and eggs of *V. mercenaria* and several other species can now be obtained on almost a year-round basis, and the larvae can be grown to the setting stage even in the middle of winter.

We wish to express our thanks to our colleague, Mr. Charles A. Nomejko, for making the microphotographs of the larvae for this article.

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THE CONTROL OF RETINAL PIGMENT MIGRATION IN LEANDER SERRATUS

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Introduction

The movements of pigments in the eyes of crustaceans are known to be related to the illumination of the eye and to the shade of the background on which the animals are placed, but the means by which these pigment movements are controlled have been disputed for more than fifty years. Conflicting evidence has pointed on the one hand to a hormonal control of these pigment movements and on the other hand to a direct response of the pigment cells to the presence or the absence of illumination. There is also the possibility that the pigment movements are under direct nervous control, but as yet no nervous connections to the distal or the reflecting pigment cells have been traced, and the proximal retinular cells containing pigment are innervated by sensory fibres only (Parker, 1891). Recent evidence has indicated that the retinal pigment cells respond to the injection of hormones, but this does not exclude the possibility that they may also respond directly to illumination, as some of the earlier evidence indicated. The present paper is an attempt to evaluate the relative importance of hormonal control and a direct response to illumination in movements of the retinal pigments in the prawn *Leander serratus*.

In 1897 Parker described experiments on *Palaemonetes vulgaris*, in which one eye was covered and the other was exposed to direct illumination. In one experiment, one eye was covered by a mixture of Canada balsam and lampblack; another experiment consisted in placing an animal in a light-proof box in such a way that one eye projected through a hole in the side of the box and was exposed to illumination. Parker reported that, in his experiments, "the eyes exposed to the light always presented the condition normal for light, and those kept in the dark always showed an approach, more or less complete, to the condition characteristic for the dark."

Experiments comparable to those of Parker were carried out by Castle (1927), who used plaster of Paris to cover the eyes of Palaemonetes and studied the effects on the movements of the proximal pigment only. Covering of one eye or of both eyes caused complete or incomplete darkness-adaption of the covered eye, although when one eye was covered and the other was exposed, the latter always showed the condition characteristic of light-adaption.

Further experiments were carried out by Bennitt (1932) on *Palaemonetes vulgaris* and *P. exilipes*, using Castle's method of covering the eye. One third of the covered eyes showed a partial adaption to darkness, but only one in forty-five of the exposed eyes of the same animals showed an intermediate state—the rest were light-adapted.

Bennitt then explored the possibility that light might be entering the covered eye through the transparent eye-stalks. To avoid this difficulty he used opaque animals, namely Cambarus, Cancer, Carcinides, and Libinia. The results, however,

were inconclusive; in some cases the illuminated eye was light-adapted and the covered eye was partially dark-adapted, while in other instances both eyes became light-adapted. Bennitt concluded that pigment movements in the two eyes of a crustacean were interrelated and that stimulation of one eye would bring about pigment migrations in both eyes; he suggested that the two eyes might be interrelated through hormonal control.

The retinal pigments of many species of crustaceans exhibit a marked diurnal rhythm of migration; in some species a diurnal rhythm persists in constant light, and in others it continues in constant darkness. The three retinal pigments behave differently in the various species. The evidence has been summarized by Brown (1944) and by Kleinholz (1949*a*). The existence of these diurnal rhythms reveals that factors other than direct illumination cause the retinal pigments to migrate. In 1930 Welsh showed that when a dark-adapted Palaemonetes was placed in the light for twenty minutes and then returned to darkness, the migrations of the retinal pigment, which began under illumination, continued for the next twenty minutes in darkness, although the stimulus for migration was no longer present. These observations led Kleinholz (1936) to investigate a possible hormonal control of distal pigment movements in Palaemonetes. His injections of eye-stalk extracts into dark-adapted animals brought about the light-adapted position of the distal and the reflecting pigments, the proximal pigment remaining unaffected by the injected extract. Welsh (1939) has subsequently shown that injections of concentrated eye-stalk extracts will cause the proximal pigment of dark-adapted Cambarus to move into the position characteristically found in light-adapted animals. It has not yet been shown that in any one species the distal, reflecting and proximal pigments can all be moved by injections of hormones, and in some species one or another of these pigments remains in a fixed position, and is unaffected by injections or by change of illumination. It seems clear, however, that the three pigments of the crustacean eye will respond to injections of eye-stalk extracts, although these injection experiments alone do not determine that the control of the pigment movements is predominantly hormonal under normal conditions. The experiments described above, which indicated a partial independence of the covered and the exposed eye of an individual, showed that other factors besides hormonal ones were involved. In the light of so much confusing evidence it seemed desirable to investigate the matter, using *Leander serratus* as a test animal.

Part of the present work was done at Plymouth and the remainder at Naples. I should like to acknowledge my gratitude to the Challenger Society which defrayed many of my expenses at Naples, and to Professor A. C. Hardy, F. R. S., who kindly allowed me to use the Oxford table there. On one occasion I occupied the table at Plymouth rented by the British Association and on another occasion that rented by the Clothworker Company. I am greatly indebted to these two bodies for the use of their tables at Plymouth. I am also indebted to the Directors and staffs of the Laboratories of the Marine Biological Association at Plymouth, and the Stazione Zoologica at Naples for their unfailing courtesy and help.

Material and Methods

In the present experiments large male individuals of *Leander serratus* were used. In order to have some means whereby the positions of the distal retinal pigment

might be expressed numerically and compared in eyes of different size, the following procedure was adopted. Measurements were taken of the distance from the basement membrane to the distal ends of the distal retinal pigment cells, and also from the basement membrane to the surface of the eye (see text Fig. 1). The position of the distal pigment can thus be expressed numerically as the ratio of the former measurement to the latter, and is denoted in the present paper as the distal retinal pigment index; this measurement provides a satisfactory method of comparing the response of the distal retinal pigment in eyes of different size.

In those experiments which required an opaque cover for the retina some difficulty was experienced in finding a satisfactory substance; one which would not dissolve in sea-water and yet did not contain a solvent that would affect the retina. In a preliminary series of experiments the mixture of Canada Balsam and lampblack used by Parker (1897) was employed, but it was found that the xylene solvent itself caused positional changes in the retinal pigments and the use of this mixture was discontinued. Eventually red modelling clay (marketed under the name of Iwana) was found to be satisfactory for covering the eyes. In applying this clay it was found convenient to secure an animal by fixing two tapes over the body, one over the cephalothorax and the other over the abdomen, and pinning these to a small board. A bit of modelling clay was fashioned into a ring which was slipped over one eye and attached firmly around the exoskeletal base of the eye-stalk; a second ring was then slipped over the eye-stalk and by using the fine point of a needle was joined to the first ring; finally, a small cap of clay was fitted over the retinal portion of the eye and moulded to the second ring around the eye-stalk. In this way a loose opaque sac of modelling clay was fitted over the eye and its stalk. The smaller diameter of the eye-stalk prevented the cover from falling off.

An alternative method of covering one eye is the box method described by Parker (1897). The animal is fastened by tapes to the inside of a light-proof box in such a way that one eye-stalk projects through a hole just wide enough to admit it. The box is then placed under water in a darkroom with a beam of light directed on the exposed eye along a plane parallel to the side of the box; this direction is calculated to allow the least possible amount of light into the interior of the box. The box experiment and the method of covering the eye with clay provide two contrasts in method. In the box method the covered eye is not constrained in any way, whereas in the other experiment it bears a clay covering. In the clay experiment the body is illuminated but in the box experiment it is not.

The intensity of light in these experiments was measured by a Weston photometer, which expressed light-intensity in terms of seconds necessary for photographic exposure. This did not give an accurate absolute measurement but was sufficiently sensitive to permit an adjustment of the source of illumination so that approximately the same intensity of light fell on the eye in each separate experiment. The temperature of the water was taken before and after each experiment. In no case had the temperature risen more than 2.0° C. at the end of a four-hour interval. Black, white and grey backgrounds consisting of painted cake tins were used in some of the experiments.

Removal of the sinus-glands in some of the experiments was accomplished by a modification of the method described by Panouse (1946). The animal to be operated upon was secured by tapes as described above, a piece of modelling wax was arranged at one side of the head and a groove was made in the wax in which

one eye could rest. The operations were generally performed in the evening after the animals had been in darkness for some hours so that the contraction of the chromatophores on the eye-stalk allowed the sinus-gland to be seen clearly through the cuticle of the stalk. The cuticle was lightly pierced above the gland with the points of fine watchmaker's forceps, and a small piece of the cuticle was removed; the aperture made was sufficiently large to allow the points of the forceps to pass through and seize the sinus-gland which is a bluish-white mass in contrast to the more translucent surrounding tissues (Fig. 2). After removal of the sinus-gland the hole in the cuticle was filled by a small plug of modelling clay which prevented any further loss of blood. In each animal excision of the second sinus-gland was performed 24 hours after removal of the first gland.

The success of sinus-gland removal was judged by (1) direct observation of the eye-stalk, (2) the behaviour of the dark pigments in the chromatophores, (3) sectioning the eye-stalks after experiment. Immobility of the dark chromatophoral pigments for ten days, under various conditions of illumination and background, was taken as an indication of successful sinus-gland removal. Later sectioning of the eye-stalks confirmed this opinion. About one-third of the operations were successful, a figure which is in agreement with those obtained by Panouse (1946) and Brown, Ederstrom and Scudamore (1939). The mortality among operated animals was about 25 per cent. In all, 148 experimental and 18 control animals were examined.

A preliminary series of experiments revealed a diurnal rhythm of activity of the proximal and reflecting pigments (Fig. 3). In constant darkness, these pigments became more completely dark-adapted at midnight than they did at midday. To avoid the extremes of this rhythm, all subsequent experiments were carried out during the afternoon and in no cases finished later than 6 p.m.

After each experiment the animals were immersed in hot water (80° C.) for 10 seconds to fix the position of the retinal pigments. The eyes were excised and transferred to a modified Bouin's solution (containing 7 per cent acetic acid) for 24 hours. They were then placed successively in 70 per cent alcohol for 3 hours, in dioxane for 12 hours and in paraffin wax for 3–5 hours. Before the final embedding, the eyes were oriented in the melted paraffin with the dorsal side uppermost (readily recognized by the black pigment-spot located at the junction of the retinal and non-retinal portions of the eye-stalk). Serial sections were cut at 15–20 μ and were generally mounted unstained for observation of the position of the retinal pigments. Measurements of the position of the distal pigment were made on the central ommatidia of those sections bearing the dorsal pigment spot; thus the measurements represent, as nearly as possible, the position of the distal pigment cells in the central ommatidia of the eye.

The Eye of Leander Serratus

The structure of the decapod eye has been described in detail by many authors; a survey of the literature on the subject has been given by Parker (1932) and a more recent account of the structure of the eyes of some species has been given by Debaisieux (1944). There is, however, a lack of uniformity in the terminology used by different authors to describe corresponding parts of the eye in different species.

The eye of *Leander serratus* is composed of about 7000 ommatidia and a dorsal accessory pigment spot. Each ommatidium (Fig. 1) comprises five cell-types as

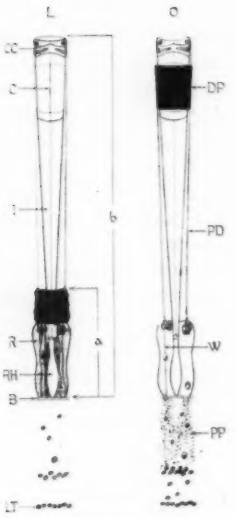


FIGURE 1.

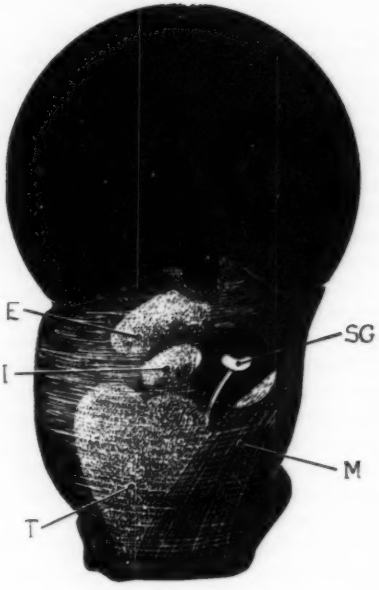


FIGURE 2.

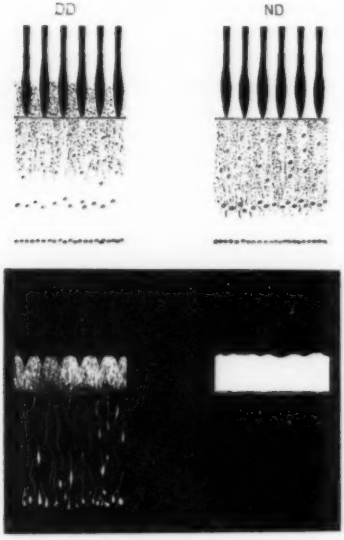


FIGURE 3.

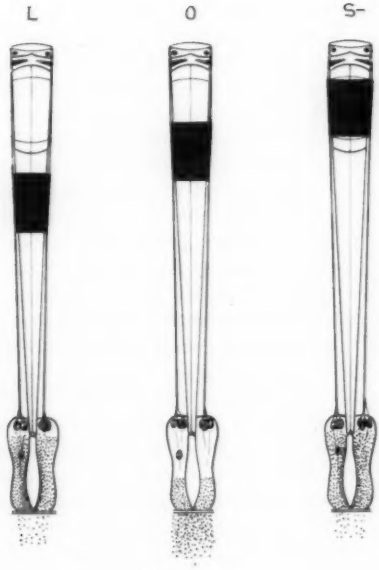


FIGURE 4.

follows: (a) Corneal hypodermal cells which secrete the cornea. (b) Cone cells which form a crystalline cone. (c) Distal retinal pigment cells. (d) Proximal pigmented retinular cells, which surround a central transparent mass, the rhabdome. (e) Tapetal pigment cells, of which there is one cell to a number of ommatidia.

The cornea is facetted in such a way that each ommatidium bears, at its distal end, one facet which is square in surface view and slightly biconvex when viewed in section. Each facet is composed of a thin scaly epicuticle and a thicker cuticle. Two corneal hypodermal cells lie beneath each facet.

The cone consists of four elements which form an elongated pyramid with its square base approximated to the proximal surfaces of the corneal hypodermal cells. In this basal portion there are four nuclei, corresponding to the four elements of the cone. Each cone element is not homogeneous throughout its length, but comprises a cell-body, the crystalline cone proper, and an extension which Debaisieux (1944) calls an intermediate crystalline tract. Distally, prolongations of the cone cell-bodies appear to pass between the corneal hypodermal cells and may come in contact with the corneal facet. Proximally the intermediate crystalline tract is prolonged into a number (Debaisieux states that there are 11 to the four crystalline tracts in *Palaemon varians*) of fine fibres which extend proximally between the retinular cells and envelop the rhabdome; in some species these fibres extend to the basement membrane, but in Leander neither Schneider (1902) nor Trojan (1913) could trace them as far as this.

The cone is surrounded by two pigment cells which have been known variously as "Hauptpigmentzellen," "Iris pigment cells," "Distal retinular cells," and "Pigmentzellen." The name "Distal pigment cell" has been widely adopted in recent literature and will be used in the present account. These distal cells contain mainly dark pigment granules, but in addition there is a thin covering sheath of white light-reflecting pigment. The distal and the proximal extensions of the distal pigment

FIGURES 1–4.

FIGURE 1. Light adaptation in the eye of *Leander serratus*. Two ommatidia are shown, the left in a condition of total light-adaptation, the right with the pigments in a dark-adapted position. The change in position of the distal and proximal pigments is depicted; the position of the reflecting pigment is not shown. The measurement a/b is described in the text as the distal pigment index.

CC. Cells of crystalline cone. C. Cone. I. Intermediate crystalline tract. R. Retinular cell. RH. Rhabdom. B. Basement membrane. LT. Lamina ganglionaris. DP. Distal pigment. PD. Proximal extension of distal pigment cell. W. Distal extension of reflecting pigment cell. PP. Proximal pigment.

FIGURE 2. The position of the sinus-gland in the eye-stalk of *Leander serratus*. The right eye is viewed from above, and a small aperture has been cut in the cuticle directly above the sinus-gland. M. Muscle. SG. Sinus-gland. E. Medulla externa. I. Medulla interna. T. Medulla terminalis.

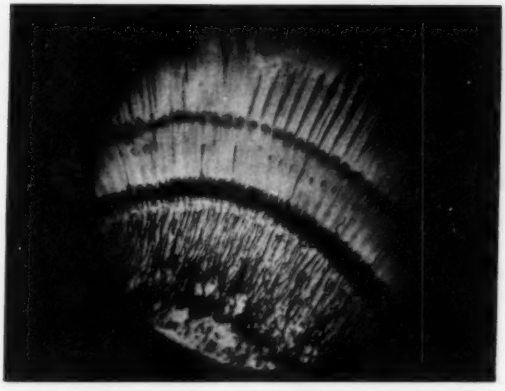
FIGURE 3. Diurnal rhythm of proximal and reflecting pigments in *Leander serratus*. The movements of the proximal pigment are shown in the upper drawings; the reflecting pigment is depicted in the lower figures. On the left, the retina of an individual killed at midday after 24 hours in constant darkness (DD). On the right, the retina of an animal killed at midnight after 12 hours in constant darkness (ND).

FIGURE 4. Illumination and the sinus-gland hormone in the movements of the distal pigment in *Leander serratus*. L and O are ommatidia from the exposed left and covered right eye respectively of an animal maintained in a box with one eye projecting through a hole in the side of the box. S- is an ommatidium from an individual from which the sinus-gland had been removed a week previously, and which had been for four hours exposed to powerful illumination.

FIGURE 5.

FIGURE 6.

FIGURE 7.



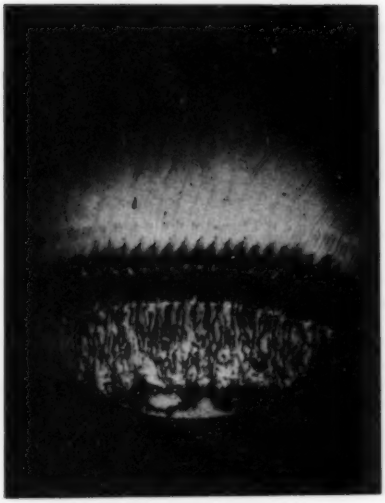


FIGURE 8.

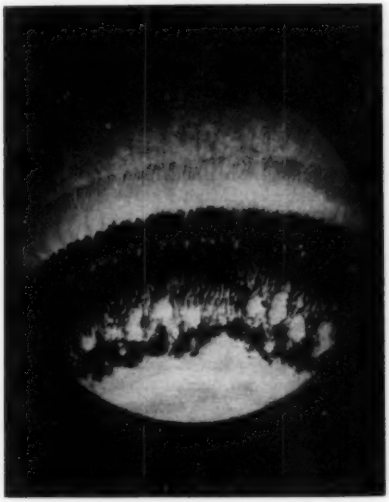


FIGURE 9.

cells have been variously described in the literature. Welsh (1930) depicted a distal extension of the pigment cells to the cornea in *Palaemonetes vulgaris* and a proximal extension by fibres to the distal ends of the retinular cells. Debaisieux (1944) shows a distal extension to form a collar around the corneal hypodermal cells and proximal fibres extending to the basement membrane. In eyes containing both dark and white pigments the connections are not easily distinguished because the white pigment appears dark by transmitted light and, moreover, it is partly masked by the dark pigment. In my preparations of retinas of *Leander serratus* I have observed that if the dark pigment is removed by controlled immersion in dilute ammonia, but the white pigment remains, the extensions described by Debaisieux can be seen (Fig. 7). If the white pigment only is removed, by immersion in boiling water, the connections depicted by Welsh can be observed. I believe that the inner darkly pigmented portion of the distal cell is in continuity with the retinular cells proximally and the cornea distally, but the sheath of white pigment is in continuity with the white pigment of the tapetal layer proximally and the cornea hypodermal cells distally.

There are eight proximal retinular cells, though one of these is very reduced in size. Proximally these cells taper to nerve fibres which pass through the basement membrane and gather to form the lamina ganglionaris, whence fibres pass to the medulla externa of the eye-stalk. The extensions of the retinular cells between the basement membrane and the lamina ganglionaris are fairly wide, and pigment contained within the retinular cells migrates above or below the basement membrane in response to the illumination falling on the eye. The pigment contained in the retinular cells is known as the proximal pigment.

The tapetal layer comprises a number of nucleated cells, about one to each ten ommatidia, which lie distally to the basement membrane, but with processes extending proximally towards the lamina ganglionaris. The tapetal cells contain a white light-reflecting pigment, and have been compared by Debaisieux to the white chromatophores of the integument; distally they extend to the distal pigment cells and beyond. The pigment they contain is referred to as the reflecting pigment in this account.

Movements of the Retinal Pigments Under Normal Conditions of Illumination

To study first the normal migrations of the retinal pigments, on illuminated backgrounds and in darkness, the following procedure was adopted: Ten large male individuals of *Leander serratus* were placed in a dish on a white background and ten

Figures 5–9.

Figure 5. The exposed and illuminated right eye of an individual maintained in a light-proof box for three hours.

Figure 6. The covered left eye of the same individual shown in Figure 5.

Figure 7. A small portion of the retina of *Leander serratus* from which the dark proximal pigment has been removed. The white reflecting pigment appears dark by transmitted light. The distal extensions of the reflecting pigment cells are shown.

Figure 8. The retina of the exposed and illuminated eye of an individual kept in a light-proof box for three hours. The proximal pigment is in the extreme light-adapted position.

Figure 9. The retina of the darkened eye of the same individual as Figure 8. The proximal pigment is in the position of greatest dark-adaptation found in any animal killed during the day.

individuals were similarly placed on a black background; both sets of individuals were exposed to the same intensity of incident illumination. A third group of ten individuals was placed in darkness. The experiment was begun at 2 p.m. and continued until 6 p.m. when the animals were killed. Welsh (1930) reported that the average time required in *Palaemonetes vulgaris* for the distal pigment of a dark-adapted individual to become completely light adapted in the light was approximately 40 minutes, while about 90 minutes was required for the reverse process of dark-adaption. Migration of the proximal pigment is more rapid than that of the distal pigment. Although observers have given different times for the various species of decapods studied (Parker 1932), in no case do these estimates exceed 2 hours. The results of illumination and background experiments on the migration of the distal pigment are given in Table I.

TABLE I

The position of the distal retinal pigment under various conditions of illumination and background. Each experiment was carried out on ten animals. I, is the intensity of incident light measured in terms of seconds necessary for photographic exposure (see methods); R, is the intensity of reflected light. The standard deviation is calculated by the formula S.D. $= \sqrt{\frac{\Sigma D^2}{n}}$.

Light intensity			Nature of background	Average distal pigment index and standard deviation
I	R	$\frac{I}{R} \times 1000$		
.04	.33	120	white	.35±.067
.04	8	5	black	.41±.01
—	—	—	darkness	.75±.006

The distal pigment *indices*, for a second series of experiments carried out under similar conditions, ranged from .27–.3 for a white background, from .5–.6 for a black background and from .75–.90 in darkness. To test the value of this distal pigment index for comparing the position of the distal pigment in different species, the figures given by Kleinholz and Knowles (1938) were calculated in the same way. Treated thus, they gave a range of from .31 to .60 for eyes of animals placed on backgrounds ranging from white to black, and exposed to such conditions of illumination that the ratio, $\frac{I}{R} \times 1000$, ranged from 9 to 62. The figure for darkness was .76. It will be seen that these figures obtained in *Leander adspersus* correspond closely to those obtained in the present experiments on *Leander serratus*. It is thus evident that in these species the distal retinal pigment migrates distally in darkness and proximally under illumination and that the shade of the background influences the position of the distal pigment.

On the other hand, the proximal pigment in the retinular cells did not show differences in animals which had been on different backgrounds under illumination, though this pigment did, however, show differences between illuminated animals and those maintained in darkness. Under illumination the proximal pigment migrated distally so that it came to lie above the basement membrane, extending along the

rhabdom as far as the nuclei of the retinular cells; in darkness a great part of the proximal pigment migrated below the basement membrane and lay between it and the lamina ganglionaris.

Persisting Diurnal Rhythm

The retinal pigments of many crustaceans show a marked diurnal rhythm of migration, which may continue in constant light or in constant darkness in different species. The literature has been reviewed by Kleinholz (1942). Inasmuch as Panouse (1946) had reported that chromatophoral pigments of *Leander serratus* migrated diurnally in constant darkness, it seemed advisable to determine whether a persisting diurnal rhythm of the retinal pigments was present in this species. Fifteen individuals were taken from a tank and placed in darkness at 9 a.m. one day. At 12 noon on that day five of these individuals were killed, and the water changed for the others; at midnight the following night five more individuals were killed and the water once more replenished; at midday the following morning the remaining animals were killed. The eyes were compared after sectioning. No significant differences in the position of the distal pigment could be observed, but the positions of the reflecting and of the proximal pigments differed in animals which had been killed at midday or at midnight (Fig. 3). The reflecting pigment of animals killed at night had migrated distally to the basement membrane and lay between the retinular cells; on the other hand, in individuals kept in darkness and killed at midday, the reflecting pigment was dispered above and below the basement membrane. A similar condition was observed in illuminated animals killed at midday. The proximal pigment of Leander in darkness killed at night had migrated proximally to the basement membrane and lay between it and the lamina ganglionaris; while the proximal pigment of animals maintained in darkness and killed at midday lay partly below and partly above the basement membrane. It did not extend distally beyond the tapetal layer (about half the distance along the length of the rhabdome). In view of this persisting diurnal rhythm of proximal and reflecting pigment movements, all experiments were conducted at the same time each day.

The effect of covering one eye

In a preliminary series of experiments carried out using Parker's (1897) method of covering an eye with a mixture of Canada balsam and lampblack, animals thus treated were maintained for four hours on an illuminated white background. The striking results obtained by Parker were confirmed, i.e., the distal pigment of the covered eye moved into the dark-adapted position while that of the exposed eye remained light adapted. But subsequent experiments showed that Canada balsam alone had an effect on the distal pigment. A minute drop of Canada balsam, without lampblack, was applied to an eye and the animal thus treated was kept under observation. In less than half an hour the distal pigment cells of the ommatidia, directly beneath the Canada balsam, had migrated towards the periphery of the eye, indicating that the positional changes observed with this method were not due to opacity of the lampblack; probably the xylene penetrated the cornea and brought about the changes in position of the distal pigment.

(*a*) *Clay method.* A series of experiments was carried out using modelling clay as an opaque covering for the eye. The position of each pigment was recorded in each experiment and is given below and in Table II(a).

TABLE II

The effect of covering one eye and exposing the other to direct, incident and reflected illumination. In animals of the (a) series the eyes were covered with modelling clay after they had been kept on white backgrounds for three hours, and they were then placed on illuminated white and grey backgrounds. Animals of the (b) series were kept for three hours in darkness before their eyes were covered with modelling clay. Animals of series (c) were kept in a light-proof box as described in the text. The standard deviation has been calculated by the formula $S.D. = \sqrt{\frac{\Sigma D^2}{n}}$.

	Numbers of animals	Conditions before experiment	Background	Time		Position of proximal pigment	Distal pigment index*
(a)	4	illumination	white	4 hours	exposed eye	light-adapted	.44±.048
					covered eye	dark-adapted	.44±.069
	6	illumination	grey	3 hours	exposed eye	light-adapted	.48±.060
					covered eye	dark-adapted	.51±.045
(b)	5	darkness	white	3 hours	exposed eye	light-adapted	.51±.027
					covered eye	dark-adapted	.60±.031
	5	darkness	black	3 hours	exposed eye	light-adapted	.59±.055
					covered eye	dark-adapted	.65±.060
(c)	3	illumination	—	2–3 hours	exposed eye	light-adapted	.49±.087
			—		covered eye	dark-adapted	.69±.098
	5	darkness	—	2–3 hours	exposed eye	light-adpated	.62±.071
			—		covered eye	dark-adapted	.69±.068

* An increase in the distal pigment index shows a movement towards the dark-adapted position.

(1) No changes were detected in the reflecting pigment.

(2) In all cases the proximal pigment of an exposed eye migrated distally in the retinular cells until it reached their distal extremities. On the other hand, the proximal pigment of the covered eye did not extend beyond the level of the tapetal layer, i.e., it occupied about half of the retinular cells above the basement membrane in each case. This is the position of the proximal pigment in animals maintained in darkness during the day. There was, therefore, a clear difference in the position of the proximal pigment in the exposed and the covered eye of each individual (Figs. 8 and 9).

(3) The positions of the distal pigments were observed under three different conditions of the animals. The first of these were animals which had been kept on illuminated white backgrounds before one of the eyes was covered, after which the animals were returned to illuminated white backgrounds. The distal pigments in these experiments showed no difference between the covered and the exposed eyes. In the second of these conditions the animals had previously been kept in darkness and one eye had been covered in very dim light. These animals were then kept on an

illuminated white background. In this case some slight difference between the covered and the exposed eyes was detected. Under the third condition the animals, previously kept in darkness, had one eye covered and were then placed on an illuminated black background. Here the difference in the position of the distal pigment in the exposed eye and the covered eye was significant. In many cases the distal retinal pigment index does not adequately represent this phenomenon, for the difference is a slight one, not easily expressed numerically, but clearly visible, especially in those animals in which the position of the distal pigment of both eyes approximated that found in darkness.

(*b*) *Box method.* The modelling clay method of covering the eye has two disadvantages: the body is exposed to illumination and light may enter the covered eye through the transparent eye-stalk. Verrier (1941) has reported that in *Leander squilla* covering of both eyes does not prevent light adaption of the distal retinal pigment if the body is exposed to illumination (her plates show that the proximal pigment of the covered eyes become partially dark-adapted like that of *Leander serratus* placed in darkness during the day). It is not surprising, therefore, that in the covered eye experiments described above the distal pigments generally became partially light-adapted.

In the box experiments, however, one eye only is exposed to illumination, while the rest of the body is in darkness. The results of these experiments are given in Table II(c). They differ from those in the previous experiments in that the distal pigment of the covered eye generally showed little or no light-adaption. The distal pigment of the illuminated eye showed a slight proximal migration which was always greater than that observed in the covered eye. (See Figs. 4, 5, and 6.)

The effect of sinus-gland removal

The effect of sinus-gland removal was studied in *Leander serratus* at Plymouth and in *Leander adspersus* at Naples. In some cases both sinus-glands were removed from an individual; in other cases the sinus-gland was removed from one eye-stalk and the other eye-stalk was ablated. The results obtained were substantially the same whichever method of sinus-gland removal was adopted. The animals were kept for ten days after the operation and then placed on a white background for 3–4 hours under powerful illumination. At the end of the experiment the animals were killed and the eyes were sectioned. A few animals without sinus-glands were kept in darkness as controls.

Migration of the proximal pigment was unaffected by the removal of the sinus-gland and invariably became light-adapted under illumination and dark-adapted in darkness. The extent of migration of this pigment was similar to that observed in normal individuals kept under similar conditions of illumination.

The position of the distal pigment in the eyes of the operated animals showed some variation in different individuals. In some individuals it attained the position of complete dark-adaptation (Fig. 4), even though the animals had been exposed to direct and powerful illumination; in some other individuals it underwent a slight proximal migration.

A few individuals of *Leander adspersus* were operated on for the removal of the sinus glands, and later, after a few days, one eye was covered with modelling clay. The results obtained by this means were similar to those obtained in *Leander serratus*

by the box method, although the migration of the distal pigment in the illuminated eye was greater than that observed in *Leander serratus*.

Discussion

The experiments which have been described in the present paper leave little room for doubt that the presence or absence of illumination has a direct effect on the cells containing pigment in the retina of *Leander serratus*. The migrations of the proximal pigment, and to a lesser degree those of the distal pigment, are greater in an exposed eye than in a covered one. It is necessary, therefore to re-examine the evidence for a humoral control of retinal pigment migration.

Evidence for the humoral control of proximal pigment migration depends on the injection experiments of Welsh (1939). An injection of eye-stalk extract caused the proximal pigment of Cambarus to migrate in darkness. It is clear from the experiments described above that the presence of the sinus-gland is not necessary for a migration of the proximal pigment in *Leander serratus* and *Leander adspersus*. Kleinholz (1948) has observed a similar persistance of proximal pigment migration after sinus-gland removal in Cambarus, and he has subsequently shown (1949b) that migrations of the proximal pigment, in response to illumination, continue in the isolated eye of Astacus in which the circulatory system no longer functions and the influence of the brain can no longer operate. The results obtained in the present experiments, by covering one eye, show that the proximal pigment responds directly to the presence or absence of illumination.

The evidence obtained from the study of persisting diurnal rhythms in *Leander serratus*, and in other species, shows that migration of the proximal pigment can take place in the absence of illumination. It is clear, therefore, that the control of the proximal pigment is a complex one, in which direct illumination is not the only factor.

The effect of injection experiments and sinus-gland removal show that the control of the migration of the distal retinal pigment is predominantly humoral. It is clear also, from the immobility of the distal pigment in certain individuals following sinus-gland removal, that the distal pigment cannot respond directly to illumination by migrating proximally. There remains, therefore, the puzzling dissimilarity between the exposed and covered eyes of single individuals in respect to their distal pigments (Fig. 4.). The present evidence suggests that light does have a direct effect on the distal pigment, but *only when the sinus-gland hormone is circulating in the blood stream*. If this is indeed the case, it is possible that light is in some way sensitizing the distal pigment cells to the hormone by which they are activated. Such a variation in the response of an effector to its activator is unusual and merits further investigation.

Summary

1. The distal and the proximal retinal pigments of *Leander serratus* can alter their positions in response to light and darkness.

2. The reflecting pigment shows a great movement at night which persists as a diurnal rhythm in continued darkness, but during the day it is unaffected by change of illumination.

3. The extent of distal pigment migration depends on the shade of the background on which the animal is placed, if the intensity of incident illumination is constant.

4. In constant darkness the proximal pigment continues to migrate diurnally for some days, at least. At midnight it shows a greater degree of dark-adaption than it does during the day; the distal pigment cells do not exhibit any detectable persisting diurnal rhythm in continued darkness.

5. After removal of the sinus-gland the distal pigment, in most of the operated animals, attained the position of maximal dark-adaption and was unaffected by change of illumination; it therefore appears not to respond directly to illumination. On the other hand, the proximal pigment in animals without sinus glands exhibited the same responses to change of illumination as those observed in normal animals.

6. In a series of experiments one eye of an animal was covered and the other eye was illuminated. In all the experimental animals so treated the proximal pigment of the exposed eye became light-adapted, but that of the covered eye attained the position of dark-adaptation observed in animals kept in darkness during the day.

7. A slight difference between the position of the distal pigment in the exposed eye and the covered eye of an individual could sometimes be observed; the difference was most striking in those animals which were on a black background, and in those animals which were placed in a light-proof box (in darkness) with one eye protruding through the box to be illuminated.

8. The distal retinal pigment of *Leander serratus* does not respond directly to illumination when the sinus gland is absent, yet in normal animals direct illumination enhances the light adaptation of the distal pigment; possibly illumination sensitizes the distal pigment cells to the hormone by which they are activated. The migration of the proximal pigment under illumination appears to be independent of the sinus gland. The influence of direct illumination on the proximal pigments appears to be great.

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